Composition, dynamics and function of extracellular polymeric substances in drinking-water biofilms

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
– Dr. rer. nat. –

vorgelegt von

Witold Dariusz Michalowski

geboren in Warschau

Biofilm Centre – Aquatische Mikrobiologie
der
Universität Duisburg-Essen

2012

Die vorliegende Arbeit wurde im Zeitraum von Januar 2008 bis August 2012 im Arbeitskreis von Prof. Dr. Hans-Curt Flemming am Biofilm Centre der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 06.11.2012

Gutachter:	Prof. Dr. Hans-Curt Flemming
	Prof. Dr. Ulrich Szewzyk
Vorsitzender:	Prof. Dr. Jochen Stefan Gutmann

“Water, taken in moderation,
cannot hurt anybody”

- Mark Twain -

ACKNOWLEDGEMENTS

First of all I would like to express my gratefulness to Prof. Dr. Hans-Curt Flemming for setting up this interesting project and for his excellent supervision throughout my research. Hans-Curt, thank you for the inspiration, as well as the trust and the liberties to implement my own ideas, which I was able to present at the very reasonable number of conferences I've attended.

Thank you to Jost Wingender for the numerous and inspiring discussions and support throughout all stages of my research.

I am deeply indebted to both, Prof. Dr. Hans-Curt Flemming and Dr. Jost Wingender, for spending countless hours reviewing this manuscript and giving creative input.

I wish to express my gratidude to Prof. Dr. Ulrich Szewzyk for accepting the task of being my co-referee.

Many thanks to the Max Buchner Research Foundation for financial support.

I would like to acknowledge Prof. Dr. Ursula Obst, Dr. Gerald Brenner-Weiß and Boris Kühl from the KIT for giving me the opportunity to identify proteins in their lab.

Thanks to Daniel, Sascha and Mathias for their assistance during my first days in the lab, as well as to Miriam for lots of advice and encouragement especially towards the end of this work. I thank Barbara for the great work investigating parts of the dynamics of drinking-water biofilms and Dominik for the incorporation experiments with *P. aeruginosa.* Thank you to all current and former colleagues who worked with me in the department of Aquatic Microbiolgy and who provided a great atmosphere and lots of fun. All of you contributed a great deal to the success of this study.

A special thanks to Silke for the support, the encouragement, the advice and the distraction whenever I needed it, as well as for proofreading this manuscript countless times. Silke, thank you for travelling the world with me!

As last, I would like to thank my family and friends who had to suffer from my lack of time, especially during the more demanding periods of this work.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

TABLE OF CONTENTS ii

LIST OF TABLES vi

LIST OF FIGURES viii

GLOSSARY xi

ABSTRACT xiii

1. INTRODUCTION 1

1.1 The biofilm way of life 1

1.2 Extracellular polymeric substances (EPS) 3

1.2.1 EPS components 6

1.2.2 EPS matrix formation 14

1.2.3 Mechanical stability of biofilms 17

1.3 Biofilms in drinking water distribution systems 19

1.4 Methods for biofilm and EPS analysis 22

1.4.1 Tools to study biofilm composition 23

1.4.2 Tools to study biofilm architecture 37

1.5 Aims of the study 41

2. MATERIALS 43

2.1 Chemicals 43

2.2 Cultivation media 46

2.3 Buffers and solutions 47

2.4 Commercial kits 51

2.5 Enzymes 51

2.6 Equipment 51

2.7 Software and databases 54

3. METHODS 55

3.1 Cultivation of drinking-water biofilms 55
3.2 Sampling of drinking-water biofilms 57
3.3 Determination of biofilm mass 57
3.4 Multi-element analysis of drinking water and biofilms 58
3.5 Scanning electron microscopy of drinking-water biofilms 58
3.6 Confocal laser scanning microscopy (CLSM) 58
3.7 Microbiological analysis 59
3.7.1 Total cell count 59
3.7.2 Heterotrophic plate count 59
3.7.3 Culturability of *P. aeruginosa* pure cultures 59
3.7.4 Culturability of *P. aeruginosa* in biofilms 60
3.8 Isolation of extracellular polymeric substances (EPS) 60
3.9 Biochemical analysis of biofilms and EPS 63
3.9.1 Proteins 63
3.9.2 Carbohydrates 63
3.9.3 Uronic acids 64
3.9.4 DNA 65
3.9.5 2-keto-3-deoxyoctonate (KDO) 66
3.10 Enzyme activity measurements 66
3.10.1 Glucose-6-phosphate dehydrogenase activity 66
3.10.2 Protease activity determination by zymogram gels 68
3.10.3 Fluorometric determination of enzyme activity 68
3.11 Molecular biology methods 70
3.11.1 Sample preparation for population analysis by DGGE 70
3.11.2 DNA Isolation 70
3.11.3 Amplification of 16S rDNA fragments 71
3.11.4 Agarose gel electrophoresis 72
3.11.5 Denaturing Gradient Gel Electrophoresis (DGGE) 72
3.11.6 Fluorescence *in situ* hybridization 73
3.12 Analysis of proteins by 2D gel electrophoresis 74
3.12.1 Sample preparation 74
3.12.2 Isoelectric focusing (IEF) 75
3.12.3 Preparation of SDS-Tris-glycine gels 76
3.12.4 Gel electrophoresis of EPS proteins 76
3.12.5 MALDI-TOF-MS 77

3.13 Gel staining and image acquisition 80
3.13.1 Coomassie Brilliant Blue staining 80
3.13.2 Ethidium bromide staining 80
3.13.3 Silver staining 80
3.13.4 Simply Blue Safe Stain 81
3.13.5 SybrGold staining 81
3.13.6 SyproRuby protein gel stain 82

4. RESULTS 83
4.1 Establishment of methods for the cultivation of drinking-water biofilms and EPS recovery 83
4.1.1 Cultivation of drinking-water biofilms 83
4.1.2 Optimization of EPS isolation by CER 85
4.1.3 Cell integrity measurements after CER isolation 86
4.1.4 Efficiency of CER isolation 88
4.2 Optimization of 2D gel electrophoresis for EPS proteins 90
4.2.1 EPS sample preparation for 2DE 90
4.2.2 Optimization of isoelectric focusing (IEF) for EPS samples 92
4.2.3 Selection of staining method for SDS-Tris-glycine gels 94
4.3 Comparison of EPS isolation methods 96
4.3.1 Quantitative comparison of EPS recovery 96
4.3.2 Qualitative comparison of isolated EPS proteins 100
4.3.3 Impact of EPS isolation methods on culturability of biofilm cells 102
4.4 Dynamics of microbial populations and biochemical composition within drinking-water biofilms 103
4.4.1 Progress of surface colonization and drinking-water biofilm formation 103
4.4.2 Dynamics of microbial populations of drinking-water biofilms over 28 d 104
4.4.3 Changes in biochemical composition of drinking-water biofilms over 28 d 106
4.4.4 2DE analysis of the dynamics of EPS proteins from drinking-water biofilms 110
4.4.5 Dynamics of proteases in drinking-water biofilms 115
4.4.6 Enzymatic activity within drinking-water biofilms 117
4.5 Comparison of drinking-water biofilms grown at different locations 119
4.5.1 Drinking water composition and characteristics at the cultivation sites 119
4.5.2 General composition of drinking-water biofilms from different locations 122
4.5.3 Microbiological and population analysis of drinking-water biofilms with different origin 123
4.5.4 Biochemical composition of drinking-water biofilms from different locations 126

4.6 Identification of EPS proteins 134
4.7 Influence of *Pseudomonas aeruginosa* on drinking-water biofilm composition 141
4.7.1 Incorporation of *P. aeruginosa* into drinking-water biofilms 141
4.7.2 Influence of *P. aeruginosa* on the biochemical composition of drinking-water biofilms 144

5. DISCUSSION 147
5.1 EPS isolation from drinking-water biofilms 149
5.2 Drinking-water biofilm formation on the elastomer EPDM 160
5.2.1 Colonization of EPDM by drinking water microorganisms 160
5.2.2 Dynamics of population diversity of drinking-water biofilms 162
5.2.3 Biochemical composition of drinking-water biofilms 164
5.3 Influence of upstream plumbing materials on drinking-water biofilms 169
5.3.1 Variability of drinking-water biofilms cultivated on EPDM according to water characteristics and plumbing materials 170
5.3.2 Incorporation of hygienically relevant microorganisms into drinking-water biofilms 176
5.4 Identification and function of extracellular proteins in drinking-water biofilms 180
5.5 Outlook 184

6. REFERENCES 187

APPENDIX 213

LIST OF TABLES

Table 1.1: Extracellular enzyme classes detected in the EPS of biofilms from various environments (adapted and supplemented from Wingender and Jaeger, 2002). 9

Table 1.2: EPS isolation procedures described in literature (adapted and supplemented from Nielsen and Jahn, 1999). 27

Table 1.3: Photometric or fluorometric assays used for the quantification of EPS components. 32

Table 1.4: Fluorescent stains or markers applied for microscopical analysis of biofilms (adapted and supplemented from Strathmann, 2002). 39

Table 2.1: Chemicals used in this study. 43

Table 2.2: Commercial kits used in this study. 51

Table 2.3: Enzymes used in this study. 51

Table 2.4: Equipment used in this study. 51

Table 2.5: Software and databases used in this study. 54

Table 3.1: Locations and drinking water installations used for the cultivation of drinking-water biofilms. 55

Table 3.2: PCR master mix composition for one reaction. 71

Table 3.3: Primers applied for 16S rDNA amplification. 72

Table 3.4: Composition of 40 % and 60 % denaturant solutions for DGGE. 72

Table 3.5: Program applied for the IEF of EPS proteins from drinking-water biofilms. 75

Table 3.6: Silver staining protocol according to Blum *et al.* (1987). 81

Table 4.1: Comparison of metal ion concentrations in drinking water and in 14 d-old drinking-water biofilm, as well as the distribution of the cations after EPS isolation by 20 min CER treatment. 90

Table 4.2: Comparison of the PCR-DGGE band patterns with regards to number of bands and similarity of profiles of drinking-water biofilms grown for up to 28 d at plumbing system A or plumbing system C. 106

Table 4.3: Number of proteins in the EPS isolated from drinking-water biofilms grown for up to 28 d in plumbing system A or plumbing system C resolved by 2DE. 110

Table 4.4: General characteristics of drinking waters at the cultivation sites of drinking-water biofilms. 121

Table 4.5: Physical characteristics of 14 d-old drinking-water biofilms cultivated at five different sites 122

Table 4.6: Inorganic composition of 14 d-old drinking-water biofilms cultivated at five different locations. 123

Table 4.7: Number of bands, number of similar bands and similarity of DGGE band patterns of 14 d-old drinking-water biofilm communities grown at five different locations and analyzed by PCR-DGGE. 125

Table 4.8: Number of protein spots detected by 2DE in the EPS of 14 d-old drinking-water biofilms cultivated at 5 different locations in 3 independent reactor runs. 130

Table 4.9: Identities of EPS proteins of 14 d-old drinking-water biofilms cultivated at plumbing system C. Score ≥ 85 indicates significant homology of mass spectra to identified proteins. 136

Table 5.1: Advantages and disadvantages of EPS isolation methods for the analysis of EPS from drinking-water biofilms. 159

LIST OF FIGURES

Figure 1.1: Model of the steps involved in biofilm formation in a drinking water pipe (modified with permission from Moritz, 2011, according to the model given by Stoodley *et al.*, 2002). .. 2

Figure 1.2: Secretion systems identified in Gram-negative and/or Gram-positive bacteria. (adapted from Tseng *et al.*, 2009). 15

Figure 1.3: Model of a biofilm formed on a surface, showing a) its structure, b) the main EPS constituents, and c) physicochemical interactions determining mechanical stability of biofilms (adapted from Flemming and Wingender, 2010). 18

Figure 3.1: a) biofilm reactor with EPDM coupons and b) experimental set up used for the cultivation of drinking-water biofilms. 56

Figure 3.2: Experimental setup used for EPS isolation by shaking in absence or presence of CER.... 61

Figure 3.3: Schematic representation of EPS isolation procedures applied for drinking-water biofilms. 62

Figure 3.4: Pipetting scheme used for the fluorometric determination of enzyme activity in a microtitre plate format. 69

Figure 4.1: Images of 14 d-old drinking-water biofilms grown on EPDM showing a) a macroscopical view of the biofilm, b) an SEM top view of a dried biofilm at 10,000 x magnification, c) and d) epifluorescence microscopic top views of live/dead stained biofilms at 100 x and 1,000 x magnification, respectively and e) a CLSM side-view (z-stack) image of the lower 60 µm of a live/dead stained biofilm at 1,000 x magnification. 84

Figure 4.2: Protein, carbohydrate and DNA concentrations in EPS of 14 d-old drinking-water biofilms after EPS isolation by shaking without (dark bars) or with (light bars) the use of CER. ... 86

Figure 4.3: Culturability of 14 d-old drinking-water biofilm cells after shaking treatment without (dark grey bars; n = 2) or with (light grey bars; n = 4) CER for up to 60 min. 86

Figure 4.4: G6PDH activity within concentrated total biofilm, as well as cell extracts and EPS obtained by 20 and 60 min of EPS isolation treatment by shaking and CER and concentrated 20 fold. 87

Figure 4.5: SEM image including an EDX spectrum and relative composition of inorganic deposits found in a dried 14 d-old drinking-water biofilm. 89

Figure 4.6: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment a) without any sample clean-up and b) after dialysis..... 91

Figure 4.7: 2DE gel of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment optimized by EPS clean-up with DNase (Benzonase) treatment and subsequent dialysis. 92

Figure 4.8: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment and focused for a) 45 kVh, b) 55 kVh, c) 65 kVh, d) 75 kVh, e) 85 kVh or f) 95 kVh. 93

Figure 4.9: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms isolated by 20 min CER treatment and stained with a) Coomassie brilliant blue (Kang *et al.*, 2002), b) SyproRuby protein gel stain or c) silver (Blum *et al.*, 1984). 95

Figure 4.10: Protein, carbohydrate and DNA concentrations in the cell fraction after EPS isolation (cells) and EPS isolated from 14 d-old drinking-water biofilms by shaking (Vortex), CER, formaldehyde/NaOH, EDTA or heat. 96

Figure 4.11: Protein, carbohydrate and DNA concentrations measured in deionized water after addition of EDTA, formaldehyde, NaOH or formaldehyde with NaOH. 99

Figure 4.12: Recovery of proteins, carbohydrates and DNA in a model EPS solutions (10 µg mL^{-1} BSA, 5 µg mL^{-1} dextran, 1 µg mL^{-1} DNA) after treatments by shaking (Vortex), CER, formaldehyde, NaOH, formaldehyde/NaOH, EDTA or heat as used for the isolation of EPS from drinking-water biofilms. 100

Figure 4.13: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by a) shaking, b) CER, c) formaldehyde/NaOH or d) heat. 101

Figure 4.14: Total cell count (TCC) and culturability of drinking-water biofilm cells before and after isolation treatments by shaking, CER, formaldehyde/NaOH, EDTA and heat. 102

Figure 4.15: Total cell count (TCC) and culturability of drinking-water biofilms grown over periods of up to 28 d in drinking water at a) plumbing system A (n = 3 independent reactor runs) or b) plumbing system C (n = 2 independent reactor runs). 104

Figure 4.16: PCR-DGGE band patterns of drinking-water biofilms grown over periods of up to 28 d in drinking-water at a) plumbing system A stained with silver (Blum *et al.*, 1987) or b) plumbing system C stained with SybrGold. 105

Figure 4.17: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and their EPS grown over periods of up to 28 d in drinking water at plumbing system A. 109

Figure 4.18: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and their EPS grown over periods of up to 28 d in drinking water at plumbing system C. 109

Figure 4.19: 2DE gels of EPS isolated from drinking-water biofilms grown on EPDM at plumbing system A for a) 5 d, b) 7 d, c) 11 d, d) 13 d, e) 18 d, f) 21 d, g)25 d or h) 28 d without the use of a protease inhibitor. 111

Figure 4.20: 2DE gels of EPS isolated from drinking-water biofilms grown on EPDM at plumbing system C for a) 5 d, b) 7 d, c) 11 d, d) 14 d, e) 18 d, f) 21 d, g)25 d or h) 28 d with the use of a protease inhibitor cocktail. 114

Figure 4.21: One-dimensional zymogram gels showing protease activity in a) drinking-water biofilms suspensions (protein load per lane: 8.5 µg) or b) EPS solutions (protein load per lane: 0.4

µg) isolated from drinking-water biofilms after cultivation for up to 28 d in plumbing system C using zymogram gels containing gelatin as substrate. 116

Figure 4.22: Total enzyme activities expressed in nmol min^{-1} of seven enzyme classes determined in the whole biofilm, in biofilm cells after EPS isolation, or in isolated EPS obtained from drinking-water biofilms after cultivation for up to 28 d. 118

Figure 4.23: Specific enzyme activities expressed in nkat mg^{-1} protein of seven enzyme classes determined in the whole biofilm, in biofilm cells after EPS isolation, or in isolated EPS obtained from drinking-water biofilms after cultivation for up to 28 d. 118

Figure 4.24: Total cell counts (TCC) and HPC of 14 d-old drinking-water biofilms grown at five different locations. 124

Figure 4.25: PCR-DGGE gel of 14 d-old drinking-water biofilm communities grown at five different locations. Arrows indicate bands at the same positions in all biofilm samples. 125

Figure 4.26: Protein-, carbohydrate- and eDNA concentrations in 14 d-old drinking-water biofilms and their EPS. 127

Figure 4.27: Proportions of isolated EPS proteins (left) and carbohydrates (right) from five different drinking-water biofilms in relation to the Cu concentration in the respective drinking water. 128

Figure 4.28: 2DE gels of EPS proteins from 14 d-old drinking-water biofilms cultivated at a) plumbing system A, b) distribution system A, c) plumbing system B, d) distribution system B and e) plumbing system C. 131

Figure 4.29: 2DE gel of EPS proteins from drinking-water biofilms. Marked spots represent excised protein spots from the 2DE gel. 135

Figure 4.30: Total cell count and HPC of drinking-water biofilms and concentrations of *P. aeruginosa* AdS before as well as 24 h and 7 d after inoculation. 142

Figure 4.31: Total cell count (TCC) and culturability of *P. aeruginosa* AdS after 24 h exposition at 20 °C to a) dilutions of drinking water in deionized water (n = 1), or b) different concentrations of copper in 6 mM phosphate buffer at pH 7.0 (n = 2). 143

Figure 4.32: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and isolated EPS, 24 h or 7 d after inoculation with *P. aeruginosa* AdS (+P.a.) or deionized water (-P.a. control) 144

Figure 4.33: 2DE gels of EPS proteins isolated from a) drinking-water biofilms before inoculation, b) drinking-water biofilm 24 h after inoculation with *P. aeruginosa* AdS in deionized water, c) drinking-water biofilm 24 h after inoculation with deionized water (control), d) drinking-water biofilm 7 d after inoculation with *P. aeruginosa* AdS in deionized water, and e) drinking-water biofilm 7 d after inoculation with deionized water (control). 146

Figure 5.10: Materials used in plumbing systems of public buildings in Germany (according to Kistemann *et al.*, 2010). 170

GLOSSARY

2DE	two-dimensional gel electrophoresis
2D-DIGE	tow dimensional differential gel electrophoresis
AAS	atomic absorption spectrometry
AFM	atomic force microscopy
APS	ammonium persulfate
ATP	adenosine triphosphate
ATR	attenuated total reflectance
BSA	bovine serum albumin
CER	cation exchange resin
cfu	colony forming units
CHAPS	[3-(3-Cholanamidopropyl)dimethylammonio]-1-propansulfonate
CLSM	confocal laser scanning microscopy
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DVGW	Deutscher Verein des Gas- und Wasserfaches (German Gas and Water Association)
EDTA	ethylene diamine tetra-acetic acid
EDX	energy dispersive x-ray
EPDM	ethylene propylene diene monomer
EPS	extracellular polymeric stubstances
ESEM	environmental scanning electron microscopy
FISH	fluorescence *in situ* hybridization
FT-IR	Fourier-transformation-infrared-spectroscopy
G6PDH	glucose-6-phosphate dehydrogenase
GC	gas chromatography
H_2CO	formaldehyde
HPC	heterotrophic plate count
HPLC	high performance liquid chromatography
ICP	inductively coupled plasma
IEF	isoelectric focusing
IPG	immobilized pH gradient

kDa	kilo Dalton
KDO	2-keto-3-deoxyoctonate ammonium salt
LB	Lenox broth
LC	liquid chromatography
MALDI	matrix assisted laser desorption/ionization
MS	mass spectrometry
MUF	methylumbelliferyl
MWCO	molecular weight cut-off
NADP	nicotinamide-adenine-dinucleotide phosphate
NMR	nuclear magnetic resonance
OES	optical emission spectrometry
p. a.	per analysis
P. a.	*Pseudomonas aeruginosa*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	polyethylene
pI	isoelectric point
PI	propidium iodide
PMMA	polymethyl-methacrylate
PVC	polyvinylchloride
RNA	ribonucleic acid
RT-qPCR	real-time quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SERS	surface-enhanced Raman scattering
SSCP	single strand conformation polymorphism
TAE	Tris/acetic acid/EDTA
TE	Tris/EDTA
TCC	total cell count
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
T-RFLP	terminal restriction fragment length polymorphism
TLC	thin-layer chromatography
TOC	total organic carbon
TOF	time of flight
TrinkwV	Trinkwasserverordnung (German Drinking Water Ordinance)
Tris	Tris(hydroxymethyl)-aminomethane
VBNC	viable but nonculturable
v/v	volume per volume
w/v	weight per volume
XTT	3'-1-[(phenylamino-)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

ABSTRACT

Drinking water distribution systems as well as domestic plumbing systems are colonized by microbial biofilms. Under unfavourable conditions they may act as reservoirs for hygienically relevant microorganisms, posing a potential threat to human health. The aim of this study was the investigation of the formation, composition and function of drinking-water biofilms and their extracellular polymeric substances (EPS). The biofilms were grown on a synthetic elastomeric material, exposed to drinking water in different drinking water distribution systems as well as public plumbing systems made of copper. Characterization of biofilms and their EPS was carried out by microbiological, molecular biological and biochemical methods, which were adapted and optimized to meet the difficulty provided by the low amounts of biomass usually found in drinking water systems.

The elastomeric material provided a suitable substratum for the cultivation of drinking-water biofilms. After 14 d of exposure, biofilm growth reached a quasi-stationary state, showing constant cell numbers in the range of 1.0×10^8 cells cm^{-2} to 5.0×10^8 cells cm^{-2}. Culturability of biofilm cells was one order of magnitude lower compared to total cell numbers. Analysis by PCR-DGGE showed an increase in population diversity for the first 7 d of biofilm growth and from then on remained constant for up to 28 d. Comparison of band patterns of biofilms in different water systems showed variable microbial composition of drinking-water biofilms, with similarities of only 46 % to 63 %.

EPS were isolated from drinking-water biofilms by application of a cation exchange resin (CER). Due to the low biomass yield this EPS isolation method was miniaturized and optimized, and compared to other commonly applied isolation methods including treatment by shaking (control), formaldehyde/NaOH, EDTA, or heat. The CER method showed significantly higher yields of EPS components compared to the control method, and, in

contrast to chemical methods or heat, caused no damage to biofilm cells or interference with analyses. Proteins represented the main component, irrespective of biofilm age or origin, followed by carbohydrates and DNA. Protein and carbohydrate contents in the EPS increased continuously throughout the cultivation period of up to 28 d, while DNA showed an increase in concentration for the initial 11 d to 21 d of cultivation, from which on the concentration remained constant. The isolation yields of EPS constituents varied depending on the biofilm's origin and showed a decreasing trend with increasing Cu content in the water phase. Similarly to protein production, also activity of the hydrolytic enzyme groups proteases, peptidases, α-/ß-glucosidases, N-acetyl-ß-D-glucosaminidases, lipases, esterases, and phosphatases increased with biofilm age, in particular once biofilms reached 14 d of age. EPS protein diversity was analyzed by two-dimensional gel electrophoresis and exhibited significant variability according to the biofilm's origin. In the course of biofilm formation, diversity of EPS proteins increased for the first 14 d and decreased from then on, showing a lower amount of protein spots with high molecular weightes or isoelectric points. Analysis of protein spots by MALDI-TOF-MS identified proteins with metabolic, transport, or regulatory functions in the EPS of drinking-water biofilms. A few protein clusters, including efflux proteins, were only present in biofilms grown in copper plumbing systems.

The incorporation of a hygienically relevant microorganism into drinking-water biofilms was examined. As a model microorganism of hygienical relevance, *Pseudomonas aeruginosa* was used to examine the hypothesis, that it can influence the composition of existing biofilms and their EPS. The incorporation of *P. aeruginosa* as detected by FISH showed the potential of drinking-water biofilms to harbor hygienically relevant microorganisms. An effect on the composition of drinking-water biofilms or their EPS was not observed.

The results demonstrated the variability of drinking-water biofilms in terms of microbial populations and EPS composition in response to variations of conditions in different distribution systems and in particular in copper plumbing systems. Furthermore, this study demonstrated the dynamics of EPS components in the course of biofilm formation, indicating continuous changes to the EPS matrix induced by the constituent organisms. Drinking-water biofilms were shown to be another type of biofilms, in which proteins represent the main EPS component, followed by polysaccharides and DNA. EPS proteins in drinking-water biofilms exhibited metabolic, transport and regulatory functions.

1. INTRODUCTION

1.1 The biofilm way of life

Biofilms represent a fascinating lifestyle found in all three domains of life and one of the oldest forms of life on earth (Flemming, 2011). The term "biofilm" describes microbial aggregates embedded in a highly hydrated, self-produced slime matrix composed of extracellular polymeric substances (EPS). EPS are composed of a variety of different macromolecules, such as polysaccharides, proteins, DNA or (phospho-) lipids, which form the three-dimensional structure of biofilms, affect their porosity, density, water content and sorption properties, and afford protection for biofilm organisms (Wingender *et al.*, 1999; Flemming and Wingender, 2002; Denkhaus *et al.*, 2007; Flemming *et al.*, 2007; Karatan and Watnik, 2009; Flemming and Wingender, 2010). Biofilms form at interfaces of two-phase systems such as water-air, water-oil, solid-air or water-solid (Wimpenny, 2000). These aggregates were investigated ever since van Leeuwenhoek's observations of the "very little animalia" he had found in the plaque on his teeth late in the 17th century. In his studies on freshwater bacteria Henrici (1933) was one of the first to describe the affinity of microorganisms to surfaces, discussing a layer of various bacteria along with algae and protozoa firmly attached to glass slides, which had been submerged into aquarium waters or Lake Alexander (Minnesota/USA). However, the term "biofilm" and its theory were not established until 1978, stating that the majority of microorganisms live as conglomerates attached to surfaces, enclosed in a self-produced matrix and exhibiting an altered gene expression compared to their planktonic counterparts (Costerton *et al.*, 1978; Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004; Karatan and Watnik, 2009). By now the biofilm mode of growth is considered the natural state of microbial existence. Over 99.9 % of microorganisms in natural aquatic environments are predicted to live in such aggregates,

suggesting that planktonic growth represents only a temporary state in microbial life (Donlan and Costerton, 2002).

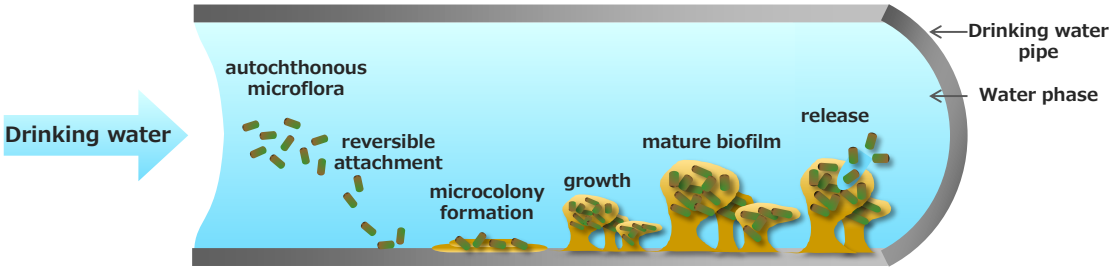

Figure 1.1: Model of the steps involved in biofilm formation in a drinking water pipe (modified with permission from Moritz, 2011, according to the model given by Stoodley *et al.*, 2002).

A generally applicable model describing the life cycle of biofilm organisms has been given by Stoodley *et al.* (2002), showing the formation and maintenance of biofilms as an on-going dynamic process (Fig. 1.1). The biofilm formation is initiated with the coating of a surface with a conditioning layer and reversible attachment of microorganisms. This initial step is strongly dependent on the surface characteristics, microorganisms involved, as well as environmental conditions. In general, high surface roughness (Percival *et al.*, 1999), as well as hydrophobicity of a substratum (Fletcher and Loeb, 1979) promotes attachment of bacterial cells. Cell density, cell surface structures such as flagella or pili, or environmental conditions such as nutrient availability, temperature, osmolarity, pH, oxygen content or hydrodynamic parameters also determine the degree of colonization of a surface (O'Toole *et al.*, 2000; Flemming and Wingender, 2001). In the second step microcolonies arise and a switch from planktonic to biofilm specific expression of genes occurs, leading to enhanced production of EPS (Watnick and Kolter, 2000; Sauer *et al.*, 2002). The microcolonies evolve into macrocolonies and form mature biofilms. Factors like nutrient limitation or physical stress can lead to sloughing off of parts of the biofilm and release of single cells. This process of detachment can be mediated by microorganisms by the production of specific enzymes, such as polysaccharide lyases, which allow these microorganisms to free themselves from the biofilm matrix (Allison *et al.*, 1998). Cells liberated from the biofilm are then transported to new locations and may result in the development of new biofilms (Sauer *et al.*, 2002). In the course of biofilm formation, microorganisms continuously alter the composition of the biofilm matrix according to their needs, which is directly influenced by environmental conditions.

The aggregation into biofilms provides various benefits for microorganisms in comparison to life in the planktonic state, which can include increased nutrient availability, protection against environmental stress such as extreme temperatures or pH, desiccation, salinity and antimicrobial agents, or enhanced gene exchange, allowing for fast adaptation to environmental changes (Hall-Stoodley *et al.*, 2004). Many of these advantageous features can be attributed to the self-produced EPS matrix surrounding biofilm cells.

1.2 Extracellular polymeric substances (EPS)

EPS represent the major constituents of microbial biofilms and determine the environmental conditions for the residing microorganisms. In a way they resemble, as Flemming *et al.* (2007) aptly called it, "the house of biofilm cells." They form the extracellular matrix of biofilms, generate their porosity, density, water content and sorption properties, and afford protection for biofilm organisms (Wingender *et al.*, 1999; Flemming and Wingender, 2002; Flemming *et al.*, 2007; Karatan and Watnik, 2009; Flemming and Wingender, 2010). Several definitions for EPS have been given in the past. Geesey (1982) for example described EPS as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates". Characklis and Wilderer (1989) defined EPS as "organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)". In general EPS are composed of a variety of macromolecules, including polysaccharides, proteins, DNA and (phospho-) lipids and can account for over 90 % of the biofilm dry weight (Wingender *et al.*, 1999; Flemming and Wingender, 2010). In early studies, polysaccharides were the main target for analysis, as they have been assumed to be the main constituents of EPS. Hence, "EPS" were formerly considered "exopolysaccharides" or "extracellular polysaccharides". However, further studies showed that proteins and extracellular DNA (eDNA) can be found in similar or even higher concentrations within EPS of environmental biofilms compared to polysaccharides (Platt *et al.*, 1985; Jahn and Nielsen, 1995; Frølund *et al.*, 1996).

Numerous functions have been ascribed to the EPS and elucidate their importance in all stages of biofilm formation. EPS are essential components during microbial adhesion to surfaces, aggregation of cells as well as cohesion of biofilms (Wingender *et al.*, 1999). They form a three-dimensional micro-environment, in which organisms are temporarily immobilised. The nature and quantity of the individual EPS components determine the architecture of a biofilm, as well as living conditions for its residents (Flemming and Wingender, 2010).

The EPS matrix is the key agent providing protection for microorganisms against environmental stress. The EPS can for instance provide nutrients for the residing microorganisms due to their ability to adsorb and store organic matter from the water phase (Decho and Lopez, 1993; Decho, 2000; Flemming, 2002). Especially in oligotrophic environments such as drinking water distribution systems, which provide only low concentrations of organic matter, this trait can be vital for the survival of biofilm organisms. The low amounts of organic substances present in the water phase can be adsorbed by hydrophobic polysaccharides and proteins, and in this way the biofilm matrix can serve as a nutrient reservoir for microorganisms (Sutherland, 2001; Leis and Flemming, 2002). Channel systems within biofilms allow for distribution of nutrients as well as metabolites into all regions of the biofilm and also allow exchange of metabolites with the surrounding bulk solution (de Beer *et al.*, 1994; Stoodley *et al.*, 1994; Costerton, 1995; Sutherland, 2001). The utilization of organic matter as nutrients can, depending on the complexity of the substances, pose a challenge for the microorganisms. A common feature of biofilms is the establishment of synergistic microconsortia in which microorganisms can engage in metabolic cooperations, allowing for degradation of very diverse and complex organic substrates as nutrient and energy source, for example during anaerobic digestion of sludge accumulated during wastewater treatment (Davey and O'Toole, 2000) or microbial nitrification (Okabe *et al.*, 1999). Extracellular enzymes play an important role in the metabolism of high-molecular polymeric substances. The EPS matrix has been shown to retain and stabilize hydrolytic enzymes, allowing for predigestion of diverse polymers and utilization of a wide variety of organic substances (Tielen, 2006). In case of nutrient deficiency, the EPS matrix itself can be utilized as nutrient source, ensuring survival of biofilm organisms (Decho and Lopez, 1993; Decho, 2000; Flemming, 2002).

The EPS matrix can, furthermore, represent a protection barrier against biocides, such as antibiotics or disinfectants, toxins, exoenzymes or toxic metals (Costerton *et al.*, 1987; de Beer *et al.*, 1994; Suci *et al.*, 1994; Wingender *et al.*, 1999; Flemming, 2002; Szewzyk and Szewzyk, 2003; Stoodley *et al.*, 2004). While planktonic cells may be relatively easily inactivated for example by disinfectants, biofilm cells embedded in the EPS matrix require more intense measures, due to different mechanisms through which they can withstand biocides (Szewzyk and Szewzyk, 2003). One mechanism for the enhanced resistance is for example reaction-diffusion inhibition of antimicrobial agents into the biofilm (de Beer *et al.*, 1994; Suci *et al.*, 1994). Chemical reactions of biocides with EPS components such as extracellular polysaccharides or with enzymes, for instance catalases degrading H_2O_2, which can be produced as stress response by inherent microorganisms, may decrease the biocide concentration up to a limit, at which it poses no threat to the microorganisms (Suci *et al.*, 1994; Watnick and Kolter, 2000). Presence of toxic metal ions has been shown to promote EPS production as means to compensate for toxic stress. The EPS components contain functional groups, such as carboxyl, amino, hydroxyl, acetyl or phosphate groups, which can interact with metal cations to form metal-complexes. In this way toxic metals are immobilised within the biofilm matrix, and thus, rendered harmless for the biofilm organisms (White and Gadd, 1998, 2000). Not only chemical reactions of biocides with biofilm components provide means for increased survival of biofilm microorganisms, also physiological and phenotypic changes occur within a biofilm, allowing for higher stress tolerance compared to planktonic cells (Gilbert *et al.*, 1990).

Moreover, EPS can protect biofilm organisms from desiccation (Roberson and Firestone, 1992; Potts, 1994). Drying events can lead to loss of intracellular water, which few single organisms can temporarily tolerate and counteract by increasing intracellular solute concentrations, for example by absorption of salts or by producing compatible solutes such as amino acids, trehalose or betaines (Measures, 1975; Harris, 1981; Lapeña *et al.*, 1987; Harland *et al.*, 2009). The biofilm matrix, however, offers the residing organisms a habitat, which withstands desiccation for prolonged periods of time. The EPS matrix acts hygroscopically, attracting and retaining water from the environment, and may cause water contents > 99 % of the biofilm wet weight (Roberson and Firestone, 1992; Flemming and Wingender, 2010). Water retention has been shown to be significantly increased in

environments if biofilms are involved. Rosenzweig *et al.* (2012) indicated that EPS components in soils can increase the water content by 270 % of its value compared to pure soil. Results by Roberson and Firestone (1992) suggested that gene expression during drying events is altered in terms of an increased polysaccharide production, in order to store increased amounts of water, and thus, enhancing the survival of biofilm organisms.

Retention of genetic information within the EPS matrix is a further characteristic of microbial biofilms. The diversity of microorganisms within environmental biofilms, which can be comprised of a variety of different bacteria or archaea, as well as algae, fungi or protozoa, results in a large and concentrated pool of genetic information, facilitating horizontal gene transfer among biofilm organisms (Flemming *et al.*, 2007; Madsen *et al.*, 2012). Transfer of genetic material among microorganisms is important for the development of new genetic traits and for the evolution of any organism. Uptake of certain genes can for example lead to the acquisition of resistance towards certain antibiotics (Fux *et al.*, 2005). Microorganisms within biofilms have been shown to exhibit accelerated rates of uptake of genetic material compared to planktonic cells (Angles *et al.*, 1993; Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003; Madsen *et al.*, 2012). The close proximity of cells and the retention of genes within the EPS matrix provide enhanced conditions for horizontal gene transfer and allow biofilm organisms to adapt faster to environmental changes compared to planktonic cells.

Hence, the EPS matrix provides biofilm organisms an ideal habitat to co-exist, interact and form long-term microcosortia.

1.2.1 EPS components

- Extracellular proteins -

Extracellular proteins are considered to have their main function as enzymes, which are associated with the cell surface or which accumulate in the biofilm matrix. Several enzyme classes have been determined in different kinds of environments, such as natural biofilms, activated sludge or wastewater biofilms (Tab. 1.1). The enzymes classes detected included polysaccharidases, proteases, lipases, esterases, peptidases, glycosidases, phosphatases,

nucleases and oxidoreductases (Wingender and Jaeger, 2002; McDougald *et al.*, 2012). The main function of these enzymes is considered to be the degradation of macromolecules or particulate matter into low molecular weight substances, which can be taken up and readily metabolised by microorganisms. This step is required as only small molecules can be transported across cell membranes into the cell. In this way organic matter sequestered from the environment by the biofilm matrix, which can substantially differ in complexity, can be utilized as nutrient source by biofilm cells. Moreover, in nutrient-limited conditions these enzymes can hydrolyse parts of the EPS matrix itself, which can then serve as nutrient source, enabling survival under starvation stress. In this case extracellular proteins display the role of an external digestive system for the microorganisms (Flemming and Wingender, 2010). Besides being of metabolic importance for the microorganisms, proteins can serve several other functions. Hydrolytic enzymes can for example be excreted by bacteria to free themselves from the biofilm once conditions become unfavourable. The enzymes degrade parts of the surrounding EPS matrix, leading to a breakdown and sloughing off of biofilm parts and liberation of the cells (McDougald *et al.*, 2012). This allows for dispersion of microorganisms into new locations and the formation of new biofilms. Dispersin B (DspB) is an example of an enzyme involved in the detachment and dispersal of biofilms. DspB is an N-acetylglucosaminidase produced by *Actinobacillus actinomycetemcomitans*, which cleaves the 1→4 glycosidic bonds of N-acetylglucosamine-containing exopolysaccharides, and thus participates in the release of biofilm cells from N-acetylglucosamine-containing EPS matrices (Kaplan *et al.*, 2003, 2004).

In addition to their enzymatic function, proteins can also exhibit structural roles within the biofilm matrix. Due to the large amount of negatively charged reactive sites, proteins are prone to engage in electrostatic cross-linkages with multivalent cations, and thus, increasing EPS stability. This type of bridging has been shown to be more pronounced among proteins than polysaccharides (Higgins and Novak, 1997; Laspidou and Rittman, 2002). Dignac *et al.* (1998) also suggested that proteins can be involved in hydrophobic bonds within the biofilm matrix. A common type of structural proteins within biofilms includes the so-called lectins. Lectins are carbohydrate-binding proteins of prokaryotic as well as eukaryotic origin, which are highly specific for certain carbohydrate binding sites (Kennedy *et al.*, 1995). Bacterial lectins are usually located on surface structures of cells, and can, in the case of pathogens,

engage in interactions with carbohydrate residues on eukaryotic host-cells or glycosylated macromolecules (Garber *et al.*, 1992). They are involved in biofilm formation and stabilization by binding to exopolysaccharides, and thus, providing an anchor between the cells and the biofilm matrix (Higgins and Novak, 1997; Tielker *et al.*, 2005). Examples of lectins include the galactose-specific LecA, the fucose-specific LecB, or the Psl-binding CdrA protein produced by *Pseudomonas aeruginosa* (Tielker *et al.*, 2005; Mayanski *et al.*, 2012).

Biofilm-associated-proteins (Bap) and Bap-like proteins represent another family of proteins with structural character. This type of proteins has first been described in *Staphylococcus aureus* biofilms. By now this family includes a wide variety of proteins, including the BapA of *Salmonella enterica*, the large adhesion protein (LapA) of *Pseudomonas fluorescence* and *Pseudomonas putida*, the enterococcal surface protein (Esp) of *Enterococcus faecalis* and AdhA adhesin of *Burkholderia cenocepacia* (Pamp *et al.*, 2007). Members of this family of proteins share several features. They are located on the cell surface, they are of high molecular weight, they contain a core domain of tandem repeats, they are involved in biofilm formation and infectious processes, and they can be mobilized (Lasa and Penadés, 2006).

A further type of structural proteins, which demonstrate important role in biofilm formation are amyloids. Amyloids are fibrous, highly insoluble and thermally as well as chemically stable proteins containing ß-sheet-rich structures with strands stacked perpendicular to the fibril axis (Nelson *et al.*, 2005; Larsen *et al.*, 2007). These structures have been known for decades in neurodegenerative diseases like Alzheimer's or Parkinson's disease. Recently they have been detected in various environmental biofilms, including freshwater biofilms, brackish water biofilms, activated sludge biofilms, as well as drinking-water biofilms (Larsen *et al.*, 2007). The high abundance of amyloids indicates their importance as EPS matrix component in environmental biofilms. Amyloids exhibit diverse functions including adhesion of microorganisms to surfaces as well as mammalian and plant cells, they mediate cell-cell interactions or provide mechanical stability for biofilms (Chapman *et al.*, 2002; Larsen *et al.*, 2008; Cegelski *et al.*, 2009; Romero *et al.*, 2009).

Table 1.1: Extracellular enzyme classes detected in the EPS of biofilms from various environments (adapted and supplemented from Wingender and Jaeger, 2002). MUF, methylumbelliferyl; XTT, 3'-1-[(phenylamino-)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate.

Enzyme	Substrate	Source	Reference
Protein-Degrading Enzymes			
Protease	Hide powder azure	Agar-grown biofilm (*P. aeruginosa*)	N. W. Ross *et al.* (1991)
		Activated sludge	
	Azocasein	River biofilm	Goel *et al.* (1998)
	River water, casein-enriched river water	River biofilm	Jones, Lock (1991)
Peptidase	L-leucine-β-naphthylamide	River biofilm,	Laurent, Servais (1995)
		Drinking-water biofilm	
	L-leucine-4-methyl-7-coumarinylamide	River biofilm	Jones, Lock (1991)
		Freshwater biofilm	Romani *et al.* (2008)
	(L-leucine-7-amido-4-methylcoumarin)	Activated sludge	Frølund *et al.* (1995)
		Marine aggregates	Hoppe *et al.* (1991)
			Smith *et al.* (1992)
			Riemann *et al.* (2000)
		Wastewater biofilm	Confer, Logan (1998)
	L-alanine-4-nitroanilide	Sewer biofilm	Lemmer *et al.* (1994)
			Teuber, Brodisch (1977)
		Activated sludge	Lemmer *et al.* (1994)
			Teuber, Brodisch (1977)
	L-leucine-4-nitroanilide	Activated sludge	Teuber, Brodisch (1977)
	4-MUF-p-guanidinobenzoate	River biofilms	Jones, Lock (1991)
			Jones, Lock (1989)
Carbohydrate-Degrading Enzymes			
Endocellulase	Cellulose	Acid mine drainage biofilm	Jiao *et al.* (2011)
	Carboxymethyl cellulose	River biofilm	Sinsabaugh *et al.* (1991)
		Acid mine drainage biofilm	Jiao *et al.* (2011)
	resorufin cellobioside	Acid mine drainage biofilm	Jiao *et al.* (2011)
Chitinase	^{3}H-chitin, ^{14}C-chitin	River and estuarine sediments	Smucker, Kim (1991)
Alginate lyase	Native alginate in biofilm	Pure culture biofilm	Boyd, Chakrabarty (1994)
		(*P. aeruginosa*)	
α-Glucosidase	p-nitrophenyl-α-D-glucopyranoside	Epilithic river biofilm	Sinsabaugh *et al.* (1991)
		Sewer biofilm	Lemmer *et al.* (1994)
		Activated sludge	Goel *et al.* (1998)
			Lemmer *et al.* (1994)
			Teuber, Brodisch (1977)

Table 1.1: *Continued*

Enzyme	Substrate	Source	Reference
	4-MUF-α-D-glucopyranoside	Stream sediment biofilm	Battin (1997)
		Lake sediment	Boschker, Cappenberg (1998)
			Mallet, Debroas (2001)
			Smith *et al.* (1992)
		Marine aggregate	Riemann *et al.* (2000)
			Confer, Logan (1998)
		Wastewater biofilm	Frølund *et al.* (1995)
		Activated sludge	
β-Glucosidase	P-nitrophenyl-β-D-glucopyranoside	River biofilm	Sinsabaugh *et al.* (1991)
			Romani, Sabater (1999)
		Freshwater biofilm	Romani *et al.* (2008)
		Biofilm from trickling biofilter	Bihan, Lessard (2000)
		Sewer biofilm	
		Activated sludge	Lemmer *et al.* (1994)
			Lemmer *et al.* (1994)
		Stream and river biofilm	Teuber, Brodisch (1977)
		Stream sediment biofilm	Jones, Lock (1989)
		Lake sediment	Battin (1997)
			Boschker, Cappenberg (1998)
			Mallet, Debroas (2001)
		Marine aggregate	Smith *et al.* (1992)
		Activated sludge	Frølund *et al.* (1995)
β-Xylosidase	p-nitrophenyl-β-D-xylopyranoside	Epilithic river biofilm	Sinsabaugh *et al.* (1991)
	4-MUF-β-D-xyloside		
		Epilithic stream and river biofilms	Jones, Lock (1989)
			Romani, Sabater (1999)
N-acetyl-β-D-glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	Epilithic river biofilm	Sinsabaugh *et al.* (1991)
		Acid mine drainage biofilm	Jiao *et al.* (2011)
	4-MUF-N-acetyl-β-D-glucosaminide	Marine aggregate	Smith *et al.* (1992)
		Freshwater biofilm	Romani *et al.* (2008)
	ELF 97-N-acetyl-β-D-glucosaminide	Activated sludge	Frølund *et al.* (1995)
		Pure culture biofilm on chitin films	Baty III *et al.* (2000)
			Baty III *et al.* (2001)
Chitobiosidase	4-MUF-β-D-N, N'-diacetylchitobioside	Marine aggregate	Smith *et al.* (1992)
β-Glucuronidase	4-MUF-β-D-glucuronide	Activated sludge	Frølund *et al.* (1995)

Table 1.1: *Continued*

Enzyme	Substrate	Source	Reference
Lipid-Degrading Enzymes			
Lipase	4-MUF-oleate	Marine aggregate	Riemann *et al.* (2000)
	4-MUF-stearate	Activated sludge	Frølund *et al.* (1995)
Esterase	4-MUF-butyrate	River biofilm	Jones, Lock (1989)
		Lake sediment	Boschker, Cappenberg (1998)
	Fluorescein diacetate	Drinking water biofilm	De Rosa *et al.* (1998)
		Sewer biofilms	Lemmer *et al.* (1994)
		Activated sludge	Frølund *et al.* (1995)
			Lemmer *et al.* (1994)
			Nybroe *et al.* (1992)
		Stream sediment biofilm	Battin (1997)
Phosphomonoesterases			
Phosphatase	p-nitrophenyl phosphate	Epilithic river biofilm	Sinsabaugh *et al.* (1991)
		Sewer biofilm	Lemmer *et al.* (1994)
		Activated sludge	Lemmer *et al.* (1994)
			Teuber, Brodisch (1977)
		Stream and river biofilms	Romani, Sabater (1999)
	4-MUF-phosphate	Marine aggregate	Smith *et al.* (1992)
			Riemann *et al.* (2000)
		Activated sludge	Van Ommen Kloeke, Geesey (1999)
	ELF-97 phosphate	Pure-culture biofilms on stainless steel	Huang *et al.* (1998)
		Activated sludge	Xu *et al.* (1998)
			Van Ommen Kloeke, Geesey (1999)
Oxidoreductase Enzymes			
Phenol oxidase	L-3,4-dihydroxy phenylalanine	River biofilm	Sinsabaugh *et al.* (1991)
Peroxidase	L-3,4-dihydroxy phenylalanine, H_2O_2	River biofilm	Sinsabaugh *et al.* (1991)
Extracellular redox activity	5-cyano-2,3-di-4-tolyl-tetrazolium chloride	Activated sludge	Wuertz *et al.* (1998)
Extracellular redox activity	Tetrazolium salt (XTT)	Activated sludge	Wuertz *et al.* (1998)

- Polysaccharides -

Polysaccharides are high molecular weight carbohydrates comprised of monosaccharides. The monomers form long chains, which are joined by glycosidic linkages. Common monosaccharides used by bacteria to produce polysaccharides include D-glucose, D-mannose, D-galactose, D-fucose, L-rhamnose, amino acids and the uronic acids D-glucuronic acid, D-galacturonic acid as well as D-mannuronic acid and L-guluronic acid. These monomers can either form homo-polysaccharides, which are composed of a single type of monosaccharide, or hetero-polysaccharides, which are built up of repeating units of several different monosaccharides. A typical example for a homo-polysaccharide is cellulose produced for example by *Acetobacter xylinum* and other bacteria belonging to the genera *Acetobacter*, *Rhizobium*, *Agrobacter* or *Sarcina* (Ross *et al.*, 1991; Kimura and Kondo, 2002). It is composed of β-1,4-glycosidic linked D-glucose monomers. Xanthan, which is produced by *Xanthomonas campestris,* is an example for a hetero-polysaccharide. Xanthan is composed of a β-1,4-glycosidic linked glucose backbone, as found in cellulose, which in addition incorporates a trisaccharide side chain of O-acetylated D-mannose, D-glucuronic acid and pyruvate-substituted D-mannose every other glucose monomer at the 3 position (Sutherland, 1990). The side chains of polysaccharides can contain organic substituents such as O-acetyl or O-succinyl esters and pyruvate ketals, or inorganic residues such as sulphate or phosphate (Sutherland, 1990; Sutherland 2001). Composition and structure and, therefore, the chemical and physical properties among polysaccharides vary greatly. Most polysaccharides are polyanionic due to the presence of uronic acids, such as glucuronic, galacturonic or mannuronic acids, or because of ketal-linked pyruvate, all of which contribute to the anionic properties of biofilms. Some, however, can also be neutral or even polycationic (Sutherland, 2001). Polysaccharides are usually very long and thin molecules with molecular weightes between 0.5 – 2 x 10^6 Da (Sutherland, 2001). Polymerases, which are located in the cytoplasmic membrane, interlink monosaccharides and form long chains of polysaccharides. These are then actively transported through the cytoplasmic membrane into the environment, for instance with the help of the polyisoprenyl-carrier (Sutherland, 1988). Some polysaccharides, such as dextrans or levans, can also be produced extracellularly. A main function of polysaccharides is the formation the three-dimensional structure of the biofilm. Cross-linkages between polysaccharide chains determine the

mechanical stability of biofilms (Mayer *et al.*, 1999; Wingender, 1999). Additional functions with regard to retention, stabilization and protection of enzymes have been attributed to polysaccharides. Tielen (2006) could show that lipases secreted by *P. aeruginosa* were retained in the EPS matrix. The enzymes formed complexes with alginate, which was a major component of the *P. aeruginosa* strain applied in the study, by docking with the positively charged amino acids arginine and histidine to the negative reactive groups of the polyanionic alginate. The lipase-alginate complexes exhibited higher heat tolerance in contrast to unbound lipase (Tielen, 2006).

- *Extracellular DNA (eDNA)* -

eDNA has been recognised as a constituent of slime produced by microorganisms under certain conditions as early as in the 1950s (Smithies and Gibbons, 1955; Catlin, 1956). Originally eDNA had been considered to be released due to leakage of the cell membrane affected by adverse environmental conditions (Smithies and Gibbons, 1955), or a residual released from lysed cells, which is retained within the biofilm matrix (Whitchurch *et al.*, 2002, Böckelmann *et al.*, 2006). Muto and Goto (1986) reported that bacteria such as *P. aeruginosa* deliberately produce eDNA, which has transforming capability. Further studies have revealed considerable amounts of eDNA in the EPS of environmental biofilms or biofilms in technical systems (Nishikawa and Kuriyama, 1968; Platt *et al.*, 1985; Frølund *et al.*, 1996; Palmgren and Nielsen, 1996). Whitchurch *et al.* (2002) provided evidence, that eDNA is an important structural component of *P. aeruginosa* biofilms, necessary for the development of a biofilm especially during the early phases of biofilm growth. Their study showed that addition of DNA degrading enzymes (DNases) to a culture medium with *P. aeruginosa* prevented biofilm formation, even though bacterial growth was not inhibited. Furthermore, up to 60 hours old biofilms of *P. aeruginosa* treated with DNase were dissolved while older biofilms showed only minor alterations to the biofilm structure. eDNA has been shown to be a universal EPS component of biofilms, and was detected for example in pure culture biofilms of *Acinetobacter calcoaceticus, Bacillus subtilis* (Lorenz *et al.*, 1991), *Neisseria gonorrhoeae* (Dillard and Seifert, 2001), *Streptococcus intermedius* (Petersen *et al.*, 2004), *S. aureus* (Rice *et al.*, 2007), *S. epidermidis* (Qin *et al.*, 2007), *Shewanella sp*. (Pinchuk

et al., 2008), *Bacillus cereus* (Vilain *et al.*, 2009), *S. mutans* (Das *et al.*, 2010), as well as environmental biofilms as found in soils or sediments (Pietramellara *et al.*, 2009) and in technical system biofilms such as activated sludge (Dominiak *et al.*, 2011). Studies by Böckelmann *et al.* (2006, 2007) revealed that eDNA was organised in distinct patterns in biofilms formed by the bacterial strain F8. This strongly supports the concept that eDNA contributes to the spatial structure of biofilms forming an extracellular filamentous network of DNA (Flemming *et al.*, 2007). Furthermore, Das *et al.* (2010) demonstrated the importance of eDNA for initial adhesion of microorganisms to surfaces. Further potential roles of eDNA in the biofilm matrix have been debated in a discussion session conducted by and summarized in Flemming *et al.* (2007). The retention of eDNA in the biofilm matrix provides indication for a facilitated gene exchange within the biofilm. It was also hypothesized that these filaments could be used for electron transfer or even communication, and that "it seemed as if the cells could move along these filaments, using them as nanowires" (Flemming *et al.*, 2007). However, these additional roles of eDNA remain speculations.

- *Lipids* -

Though often disregarded in EPS analysis, lipids can also constitute a significant portion of microbial EPS. Gehrke *et al.* (1998) as well as Sand and Gehrke (2006) for example identified these polymers to be a major EPS component and crucial for adhesion of *Thiobacillus ferrooxidans* to pyrite surfaces. Phospholipids, glycolipids and neutral lipids have also been described as components of activated sludge flocs (Conrad *et al.*, 2003).

1.2.2 EPS matrix formation

The release of EPS components into the matrix plays a central role in the formation of biofilms. The release can occur actively, by secretion of the macromolecules through the inner and outer membranes of a cell, by formation and release of membrane vesicles, or passively through cell death and consequential discharge of intracellular material into the surroundings.

- Active secretion -

Six active secretion mechanisms have been identified for Gram-negative bacteria, which involve complex arrangements of numerous secretory proteins bound to the inner or outer cell membrane or spanning across the periplasm, and which form specific pathways for the transport of polymers across the membranes (Hueck, 1998; Russel, 1998; Abdallah *et al.*, 2007; Fronzes *et al.*, 2009). The main features of the different secretion systems are depicted in Fig. 1.2. Type I, type II and type V systems secrete polymers into the environment, where they can be directly incorporated into the EPS matrix. Type I systems have been identified as pathways for the secretion of proteins as well as polysaccharides (Bliss and Silver, 1996; Drummelsmith and Whitfield, 2000; Tseng *et al.*, 2009), whereas type II and type V systems have only been described to release proteins (Chen *et al.*, 2009; Tseng *et al.*, 2009). Type III, type IV and type VI pathways, on the other hand, mediate microbe-host interactions, for example transferring effector proteins or virulence factors, which may modify host cell functions or, in case of type IV systems, be involved in the exchange of genetic material (Tseng *et al.*, 2009). Despite the differences in membrane structure, Gram-positive bacteria have developed some of the same secretory pathways as found in Gram-negative bacteria. Additionally, an alternative type VII system specific for Gram-positive bacteria has recently been described for *Mycobacteria* and *Streptomyces coelicolor*, allowing for translocation of macromolecules through their hydrophobic and hardly permeable cell wall (Abdallah *et al.*, 2007; Tseng *et al.*, 2009; San Roman, 2010).

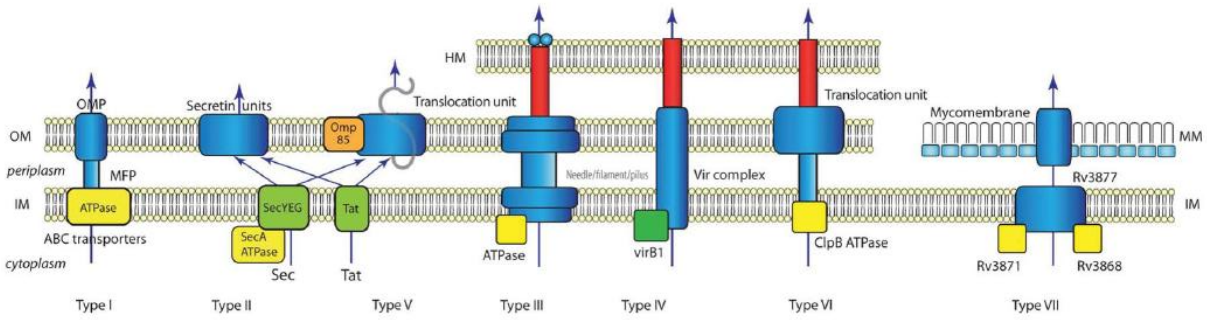

Figure 1.2: Secretion systems identified in Gram-negative and/or Gram-positive bacteria. HM: host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein (adapted by permission from BioMed Central Ltd: Tseng *et al.*, 2009).

- Membrane vesicles -

Membrane vesicles represent a further export mechanism for polymers, such as proteins, polysaccharides, nucleic acids, lipopolysaccharides or phospholipids, which is found in Gram-negative bacteria (Kuehn and Kesty, 2005) and archaea (Ellen *et al.*, 2009). Membrane vesicles, in case of Gram-negative bacteria, are spherical lipid bilayers composed of the cell's outer membrane. The outer cell membrane forms a bleb which incorporates periplasmic constituents and which is then released into the environment. In case of archaea, this bleb is composed of tetraether lipids, which are coated with an S-layer (Ellen *et al.*, 2009). This type of secretion has been described for a number of organisms, often of pathogenic character, including *E. coli*, *P. aeruginosa*, *Campylobacter jejuni*, *Helicobacter jejuni*, *Salmonella* spp., *Shigella* spp., *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii* (Hoekstra *et al.*, 1976; Logan and Trust, 1982; Kadurugamuwa and Beveridge, 1995; Fiocca *et al.*, 1999; Kandurugamuwa and Beveridge, 1999; Vesy *et al.*, 2000; Kuehn and Kesty, 2005; Ellen *et al.*, 2009). Pathogens have been shown to use this type of secretion to engage in malignant host interactions (Kadurugamuwa and Beveridge, 1995; Beveridge *et al.*, 1997; Wingender *et al.*, 1999; Kuehn and Kesty, 2005). A variety of polymers has been detected within membrane vesicles, including hydrolases, proteases, phosphatases, adhesins and toxins (Kuehn and Kesty, 2005; Ellen *et al.*, 2009). Due to their structure, membrane vesicles contribute to the lipopolysaccharide and/or protein fraction of the EPS and play a potential structural role within the matrix (Schooling and Beveridge, 2006).

- Cell lysis -

Cell death and consequential lysis of cells is a common and necessary element for the formation of biofilms and represents another mechanism of polymer discharge (Rice *et al.*, 2007; O'Connelll, 2007). This type of release can represent a strategic mechanism for fast release of the entire intracellular material into the environment as reaction to environmental changes, regulated by a genetic program. Bacterial programmed cell death has first been reported in *E. coli* in late 20th century (Naito *et al.*, 1995; Yarmolinsky, 1995). The best studied systems for programmed cell death are toxin-antitoxin modules, classified into the eight so far identified families *ccd, relBE, parDE, higBA, mazEF, phd/doc, vapBC/vag*,

and ω–ε–ξ, which are highly abundant among bacteria as well as archaea (Gerdes *et al.*, 2005; Pandey and Gerdes, 2005; Engelberg-Kulka *et al.*, 2006). "Normal" environmental conditions allow for a simultaneous expression of genes encoding for both, the toxin and the corresponding antitoxin, inhibiting the action of the toxin. Stress conditions, however, result in the deliberate arrest of expression of both genes. Since the antitoxin is more labile than the toxin, the action of the toxin is no longer inhibited and growth arrest or cell death occurs (Engelberg-Kulka *et al.*, 2006). For single cells these mechanisms appear disadvantageous, however, biofilm communities can highly benefit from the death of a subpopulation (Webb *et al.*, 2003). Starvation for instance can trigger programmed cell death of a subpopulation, providing nutrients for the remaining population, and thus, enabling their survival (Engelberg-Kulka and Hazan, 2003). Recent studies indicated that programmed cell death may be a mechanism important for biofilm formation, which enables the release of structural EPS components, in particular DNA, into the biofilm matrix (Rice *et al.*, 2007; O'Connell, 2007). Furthermore, dispersion of cells from mature biofilms can be induced by the death of a subpopulation, releasing EPS degrading enzymes, and thus, liberating cells from the matrix (Garcia-Contreras *et al.*, 2008; Rice *et al.*, 2009; Sillankorva *et al.*, 2010; McDougal, 2012).

1.2.3 Mechanical stability of biofilms

Environmental or industrial biofilms often encounter external perturbations in the form of mechanical forces such as shear. Despite their slimy appearance, biofilms exhibit impressive resilience towards these forces, while offering an adequate habitat for microorganisms to reside. The EPS composition determines mechanical stability of biofilms. Macromolecules like polysaccharides, proteins, DNA and lipids interact with themselves or with one another and form a complex three-dimensional network, which enforces cohesion and adhesion of the biofilm (Fig. 1.3; Flemming and Wingender, 2010). Multivalent cations can further enhance cross-linkages of otherwise repelling, negatively charged reactive groups of polymers (Busch and Stumm, 1968; Steiner *et al.*, 1976; Mayer *et al.*, 1999; Körstgens *et al.*, 2001).

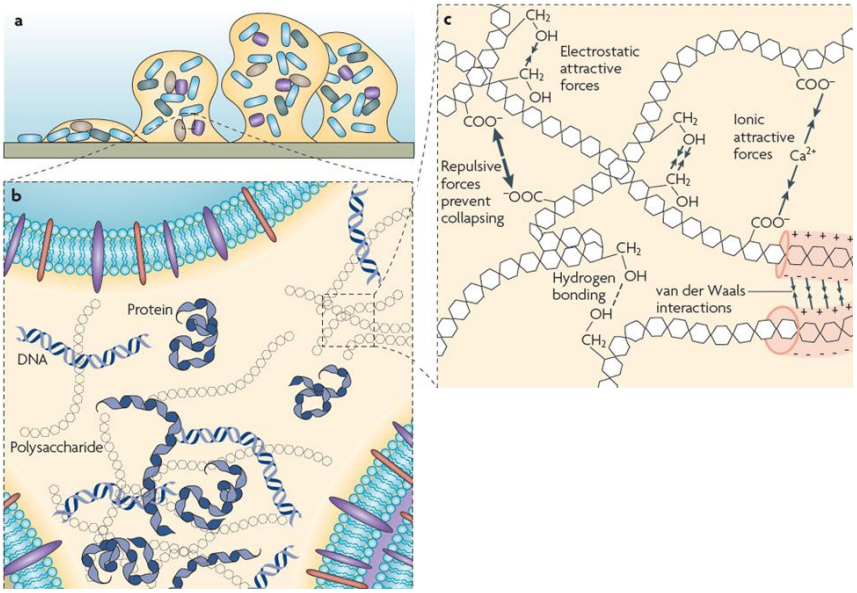

Figure 1.3: Model of a biofilm formed on a surface, showing a) its structure, b) the main EPS constituents, and c) physicochemical interactions determining mechanical stability of biofilms (adapted by permission from Macmillan Publishers Ltd: Flemming and Wingender, 2010).

Three types of forces play the major role for the cohesion of macromolecules in biofilms (Mayer *et al.*, 1999). London forces can occur in nonpolar molecules if their electron density is unevenly distributed. This results in the formation of a temporary multipole, which causes attraction. The binding energy is approximately 2.5 kJ mol^{-1} but can be weakened by surface-active substances. Electrostatic interactions are further types of binding forces formed between ions or permanent and induced dipoles. Multivalent cations, such as Ca^{2+} or Mg^{2+}, are responsible for relatively strong interactions. The binding energy ranges between 12 and 29 kJ mol^{-1} depending on the distance between the binding partners. Ionic strength, complexing agents as well as acids or bases can influence electrostatic interactions (Mayer *et al.*, 1999). The third major type of bonds, which are active in the biofilms, are hydrogen bonds. This type of interaction exists between an electronegative atom and a hydrogen atom bound to another electronegative atom. In the biofilm matrix this type of bonding often occurs between hydroxyl groups, as found in polysaccharides, and water molecules. The binding energy ranges between 10 and 30 kJ mol^{-1}. Chaotropic agents such as urea, tetramethyl urea or guanidine hydrochloride can influence these forces (Flemming and Wingender, 2003).

These three types of bonds show relatively low binding forces compared for example to covalent bonds. Due to the large amount of binding sites, however, the total binding forces may result in equivalent and even higher values compared to covalent bonds (Mayer *et al.*, 1999; Flemming and Wingender, 2003).

1.3 Biofilms in drinking water distribution systems

Biofilms are ubiquitous in environmental as well as technical systems in contact with water. Drinking water distribution systems offer diverse surfaces, which are common targets for biofilm formation. Starting from the extraction point of raw water, biofilms can be found throughout the drinking water production chain, including inner walls of pipes, the different stages of water treatment, valves or water storage tanks, all the way to the consumer's tap (Kilb *et al.*, 2003; Schmeisser *et al.*, 2003; Schwartz *et al.*, 2003; Bagh *et al.*, 2004; Emtiazi *et al.*, 2004; Wingender and Flemming, 2004; Hoefel *et al.*, 2005; Bressler *et al.*, 2009; Moritz *et al.*, 2010; Waines *et al.*, 2011; Wingender, 2011; Flemming *et al.*, 2012). The extent and microbiological and biochemical composition of these biofilms can vary greatly depending on the water source, organisms present, colonized surface or environmental conditions such as pH, water temperature, hydrodynamic parameters, available nutrients or disinfectants (Roeder *et al.*, 2010a; Flemming *et al.*, 2012). Flemming *et al.* (2002, 2012) estimated that 95 % of the overall biomass within drinking water systems can be found attached to surfaces. Due to its oligotrophic conditions drinking water systems permit only limited biofilm development and growth occurs until a plateau phase is reached, which does not interfere with water quality. This plateau phase is controlled by factors like shear forces, temperature and nutrients (Flemming, 2011). A number of studies have investigated the extent of biofilm formation on pipe surfaces of drinking water distribution systems or on coupons submerged in drinking water. These studies determined total cell counts in the range of 10^4 and 10^8 cells cm^{-2}, while culturability was significantly lower between 10^1 and 10^6 cfu cm^{-2} (LeChevallier *et al.*, 1987; Block *et al.*, 1993; Kalmbach *et al.*, 1997; Wingender and Flemming, 2004; Långmark *et al.*, 2005a; Moritz *et al.*, 2010). Once availability of nutrients is increased, for example through insufficient drinking water treatment or when materials containing biodegradable ingredients are implemented, excessive biofilm growth can occur and lead to

sloughing off of biofilm parts with subsequent contamination of the water phase (Flemming, 2002). In a case study Kilb *et al.* (2003) identified rubber coated valves as potential point sources for continuous contamination of drinking water in different drinking water distribution systems in north-west and north Germany. The surfaces of these valves, which were made of ethylene-propylene-diene monomer (EPDM) rubber, inhabited elevated numbers of microorganisms with total cell counts between 10^6 and 10^9 cells cm^{-2}. EPDM rubber materials are frequently used in drinking water distribution systems, for example for valves or sealings. However, it has been shown that certain formulations of these synthetic materials leach considerable amounts of nutrients, which can be readily utilized by microorganisms and, thus, lead to significant biofilm development (Rogers *et al.*, 1994; Kilb *et al.*, 2003).

According to the German Drinking Water Ordinance, drinking water is supplied in food-quality and water suppliers need to guarantee chemically and microbiologically stable drinking water up to the water meter of a plumbing system. However, by the time the water reaches the consumer's tap, the water can be substantially altered in quality compared to the water leaving the water works (Pepper *et al.*, 2004; Flemming *et al.*, 2012). Since drinking water distribution systems are systematically monitored, beginning from the abstraction point up to the water meter, the major source of concern remain public and domestic plumbing systems. Plumbing systems are, according to regulations, responsibility of the owner of a building (TrinkwV, 2011). The materials used for plumbing systems can differ to those used in drinking water distribution systems and include elastomers, synthetic materials, copper or stainless steel (Kistemann *et al.*, 2010). Also materials may be implemented, which are not suitable or unapproved for the use in drinking water systems (Schauer *et al.*, 2008; Kistemann *et al.*, 2010; Flemming *et al.*, 2012). Increased biofilm development may be supported due to the wrong choice of material. Auxiliary equipment, such as water softeners, filters, or phosphate dosage devices, has also been shown to increase the risk of enhanced biofilm formation (Völker *et al.*, 2010). Conditions within household installations can differ significantly from those in a water distribution system. Domestic installations can include dead ends, resulting in points of stagnating water, and also longer retention times of water are not uncommon, for example during vacations, both of which allow for biofilm formation under low shear stress (Lautenschlager *et al.*, 2010;

Flemming *et al.*, 2012). Water temperature can be elevated in plumbing systems compared to the drinking water distribution systems, due to the pipe's passage of heated rooms or close proximity to warm water systems, especially if the pipe's insulation is poor (Flemming *et al.*, 2012). These factors can result in conditions favouring a different biofilm flora.

Biofilms in drinking water distribution or plumbing systems are mainly formed by autochthonous microorganisms, which are generally harmless to human health (Wingender, 2011; Flemming *et al.*, 2012). This includes members of the groups of *α-, ß-, and γ-Proteobacteria,* as well as *Actinobacteria, Bacteroides, Firmicutes or Nitrospirae* (Schmeisser *et al.*, 2003; Hoefel *et al.*, 2005; Eichler *et al.*, 2006; Bai *et al.*, 2010; Yu *et al.*, 2010). However, recent studies have demonstrated the potential of drinking-water biofilms to accommodate hygienically relevant microorganisms, which under unfavourable conditions can pose a threat to human health (Szewzyk *et al.*, 2000; Flemming *et al.*, 2002; Bressler *et al.*, 2009; Moritz *et al.*,2010). Hygienically relevant microorganisms such as coliform bacteria, which are indicators of faecal contamination, have been shown to reside in these biofilms (Flemming *et al.*, 2002; Kilb *et al.*, 2003; Wingender and Flemming, 2011). Also opportunistic pathogens like *P. aeruginosa* or *Legionella pneumophila* (Rogers *et al.*, 1994; Flemming *et al.*, 2002; Bressler *et al.*, 2009; Moritz *et al.*,2010), which are known to be involved in severe cases of pneumonia, as well as *Aeromonas, Campylobacter, Cryptosporidium, Helicobacter, or Mycobacterium* species (Berry *et al.*, 2006; Yu *et al.*, 2010; Wingender, 2011), or viral pathogens (Skraber *et al.*, 2005; Berry *et al.*, 2006; Wingender and Flemming, 2011) can be incorporated and reside in biofilms for prolonged periods of time. The influence of these microorganisms on biofilm or EPS composition, however, has not been investigated so far.

Microbiological requirements for drinking water quality are based on cultivation on diverse media, aiming at the determination of indicator parameters, such as colony counts of heterotrophic microorganisms, or microbiological parameters, such as presence of culturable coliform bacteria, *Escherichia coli* and enterococci, as well as *P. aeruginosa* or the recently added *Legionella* spec. within the water phase (TrinkwV, 2011). Cultivation techniques, however, are selective for certain microorganisms and, therefore, do not reflect the total population of a microbial community (Amann *et al.*, 1995; Moritz *et al.*, 2010; Wingender, 2011; Wingender and Flemming, 2011). The vast majority of microorganisms cannot be cultivated on standard media and, moreover, adverse environmental conditions

may induce the transition of microorganisms into a viable but nonculturable (VBNC) state, resulting in an underestimation of the total microbial flora (Kalmbach *et al.*, 1997; Wingender, 2011). The VBNC state is defined as a state, in which bacteria "fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity" (Oliver, 2005) and has been of particular interest in recent studies investigating biofilms in drinking water systems. Moritz *et al.* (2010) showed that *P. aeruginosa* can be incorporated and survive for prolonged periods in drinking-water biofilms, and indicated that *P. aeruginosa* may enter the VBNC state if grown in installations made of copper. Dwidjosiswojo *et al.* (2011) confirmed the transition into the VBNC state due to copper, showing complete inactivation of *P. aeruginosa* in 10 µM copper solutions and successful resuscitation once copper ions were complexed by diethyldithiocarbamate. This state poses a risk for human health, since *P. aeruginosa* would remain undetected by routine drinking water analyses, especially in plumbing systems, which often include copper materials. Microbial growth on the surfaces of drinking water installations in the form of biofilms is not considered by routine examinations. However, biofilms may serve as a reservoir for hygienically relevant microorganisms and can be reason for continuous contaminations with on a routine basis undetected pathogens (Wingender, 2011).

1.4 Methods for biofilm and EPS analysis

The analysis of biofilms and their EPS represents a challenging field of research. There is no single method able to completely characterize the different facets of a biofilm. Instead interdisciplinary work is required, including microbiology, molecular biology, biochemistry, physical chemistry, analytical chemistry, engineering and modelling to be able to thoroughly investigate the nature of biofilms. Various techniques have been developed or adapted for biofilm research, aiming at the identification of the microbial or (bio-) chemical composition, the architecture or processes occurring within a biofilm (Denkhaus *et al.*, 2007).

Biofilms are rarely investigated in detail in their natural habitat. The usual case is to recreate environmental biofilms in the laboratory under standardized conditions or preferably simulating growth conditions found in the biofilm's native environment. Biofilm analysis,

therefore, begins with the cultivation of model biofilms, adjusting temperature, pH, substratum, nutrients or other physicochemical properties to produce true-to-life conditions. Flow cells or reactors, or batch systems, such as microtitre plates or fermenters are commonly applied in the laboratory as means for biofilm studies (Denkhaus *et al.*, 2007). A number of systems have been developed to investigate biofilm formation by drinking water organisms, including the rotating disc reactor (Murga *et al.*, 2001), the Propella^{TM} reactor (Appenzeller *et al.*, 2001), glass flow-through reactors (Bressler *et al.*, 2009), or stainless-steel flow-through reactors (Moritz *et al.*, 2010), or units, which can be directly incorporated into laboratory-scale test systems as well as full-scale drinking water distribution systems, such as the Robbins device (Manz *et al.*, 1993), the Bioprobe monitor (LeChevallier *et al.*, 1998), the Prevost coupon (Prévost *et al.*, 1998), the sliding coupon holder (Chang *et al.*, 2003), the biofilm sampler (Juhna *et al.*, 2007) or the Pennine Water Group (PWG) coupon (Deines *et al.*, 2010). These systems allow for insights into the different aspects of biofilm life.

1.4.1 Tools to study biofilm composition

Biofilms are heterogeneous agglomerations of microbial cells embedded in a highly hydrated matrix of EPS. The most basic parameters applied for biofilm characterization are gravimetrical determinations of the biomass' wet and dry weight, total cell count or determination of the culturability of biofilm organisms on cultivation media, and give a general overview of the formed biomass. More advanced methods for identification and quantitation of biofilm organisms, as well as the polymers, which constitute their EPS matrix give detailed information important for understanding of structural, functional or ecological aspects of biofilm existence, and are presented in this section.

- Population analysis –

Molecular biology techniques allow for cultivation-independent characterization of multi-species biofilm communities, omitting the cultivation bias on standard cultivation media.

Denaturing gradient gel electrophoresis (DGGE) is the most widely applied method to analyse population diversity. This method separates polymerase chain reaction (PCR)-amplified 16S rDNA fragments isolated from multi-species communities based on their guanine/cytosine content-mediated resistance to denaturation, and thus, provides a diversity fingerprint for a specific biofilm community (Muyzer *et al.*, 1993). DNA-bands excised from DGGE-gels can be further processed for sequencing to identify the corresponding microbial species. DGGE has been applied in a number of drinking-water biofilm related studies. Emtiazi *et al.* (2004) as well as Hoefel *et al.* (2005) investigated the community composition of drinking-water biofilms during different stages of drinking water treatment. Bressler *et al.* (2009) analyzed the population diversity of biofilms cultivated on EPDM coupons, which were inserted into glass reactors and continuously perfused with drinking water from a plumbing system. Deines *et al.* (2010) examined dynamics in population diversity in drinking-water biofilms grown for up to 11 days in a model drinking water distribution system. DGGE analysis also revealed that the choice of plumbing material as well as long-term disinfection have a significant impact on the composition of the biofilm community (Roeder *et al.*, 2010a,b). Hence, DGGE is a powerful tool to examine the microbial diversity of complex mixed-culture biofilms, as well as population shifts in response to altered environmental conditions or stress. Along with DGGE, methods like terminal restriction fragment length polymorphism (T-RFLP) (Martiny *et al.*, 2003; Pavissich *et al.*, 2010), single-strand conformation polymorphism (SSCP) (Eichler *et al.*, 2006) or intergenic transcribed spacer (ITS) region analysis (Pozos *et al.*, 2004) are potential molecular biological methods to study microbial diversity in biofilms, and which have been employed in population analyses in drinking water systems.

Other molecular biology techniques, like real-time quantitative PCR (RT-qPCR) or fluorescence *in situ* hybridization (FISH) are capable to specifically target groups of organisms of choice within a mixed culture sample. RT-qPCR employs a similar strategy as conventional PCR, using species-specific primers in combination with a fluorescent DNA marker, which allows for simultaneous quantitative measurement of the amplification, as well as inference of the initial concentration of the target gene sequence in a sample. FISH involves fluorescently-labeled oligonucleotide probes complementary to a specific DNA or RNA region of the target organism, which selectively bind to, and visualize the target. Both

techniques have been shown to allow detection and quantification of hygienically relevant microorganisms in the drinking water environment, including the groups of Proteobacteria, *Legionella* spp. or *P. aeruginosa* (Manz *et al.*, 1992, 1993, 1995; Kalmbach *et al.*, 1997; Batté *et al.*, 2003; Schwartz *et al.*, 2003; Fiume *et al.*, 2005; Wellinghausen *et al.*, 2005; Wullings and van der Kooij, 2006; Declerck *et al.*, 2009; Mathieu *et al.*, 2009; Moritz *et al.*, 2010). In addition to the quantitative assessment, FISH can be applied to investigate spatial localization of the target organism within a fully hydrated biofilm matrix (Schramm *et al.*, 1996; Manz *et al.*, 1999; Okabe *et al.*, 1999; Nielsen *et al.*, 2000; Thurnheer *et al.*, 2004; Okabe *et al.*, 2005; Bressler, 2008).

- EPS isolation -

The common approach for the analysis of EPS requires a separation of the EPS matrix from the embedded microorganisms. The isolation of EPS is a crucial step for the analysis of biofilms and their EPS, since the efficiency of the isolation method influences the outcome of any subsequent analysis. An ideal isolation technique would be one that extracts the complete fraction of EPS from biofilms, while disruption of cells, which would lead to contamination of EPS with intracellular components, is completely avoided. Such a technique does not exist, hence, EPS isolation remains a compromise of maximizing the yields of isolated EPS while detrimental impact on biofilm cells is kept at a minimum. The EPS isolation method needs to overcome the binding forces within the biofilm for a thorough separation of EPS from cellular material. The aim is to destabilize the EPS matrix through application of a physical force, leading to the break of chemical bonds, or by addition of chemicals, to remove cross-linking multivalent ions and/or to cause a pH shift, which weakens the binding forces within the EPS and helps to solubilize its components. Commonly used EPS isolation techniques include chemical methods, e.g. treatment with NaOH (e.g. Brown and Lester, 1980; Karapanogiotis *et al.*, 1989; Tapia *et al.*, 2009), formaldehyde (e.g. Liu and Fang, 2002), EDTA (e.g. Platt *et al.*, 1985; Liu and Fang, 2002; Tapia *et al.*, 2009), or crown ether (e.g. Wuertz *et al.*, 2001; Aguilera *et al.,* 2008), physical methods, such as (high-speed) centrifugation (e.g. Brown and Lester, 1980; Wingender *et al.*, 2001; Liu and Fang, 2002), sonication (e.g. Brown and Lester, 1980; Azeredo *et al.*, 1999), filtration (e.g. Hejzlar

and Chudoba, 1986), or heating/boiling (e.g. Brown and Lester, 1980; Forster and Quarmby, 1995) or combinations of chemical and physical methods, in particular the use of a cation exchange resin (CER) (e.g. Jahn and Nielsen, 1995; Frølund *et al.*, 1996) (Tab. 1.2). These techniques have been applied over past decades in a number of studies, mostly dealing with pure culture biofilms or activated sludge (Nielsen and Jahn, 1999; Liu and Fang, 2003; Sheng *et al.*, 2010). Possible cell lysis induced by the isolation methods has often been disregarded, even though this can substantially alter and falsify results (Nielsen and Jahn, 1999).

Table 1.2: EPS isolation procedures described in literature (adapted and supplemented from Nielsen and Jahn, 1999). n. d., not determined; G6PDH, glucose-6-phosphate dehydrogenase; KDO, 2-keto-3-deoxyoctonate; PS, polysaccharide.

Method	Sample	Indicator for cell lysis	Reference
Physical isolation procedures			
Centrifugation (5000 – 10 000 x g)	Pure culture biofilm (*Azosporillum brasilense*)	n. d.	Troch *et al.* (1992)
Centrifugation (5000 – 13 000 x g)	Pure cultre biofilms (*Pseudomonas putida, P. fluorescens*)	n. d.	Conti *et al.* (1994)
	Pure culture biofilm (*P. alcaligenes*)	n. d.	Titus *et al.* (1995)
	Pure culture biofilm (*Rhodopseudomonas acidophila*)	DNA	Sheng *et al.* (2005)
	Activated sludge	DNA	Gehr and Henry (1983)
Filtration	Activated sludge	n. d.	Hejzlar and Chudoba (1986)
Heating/boiling	Pure culture biofilm (*Proteus vulgaris*)	n. d.	Schmidt and Ahring (1994)
	Pure culture biofilm (*R. acidophila*)	DNA	Sheng *et al.* (2005)
	Activated sludge	DNA + proteins	Brown and Lester (1980)
		n. d.	Morgan *et al.* (1990)
		n. d.	Beccari *et al.* (1980)
		DNA	Comte *et al.* (2006)
	Digested sewage sludge/activated sludge	DNA	Forster and Quarmby (1995)
	Digested sewage sludge	n. d.	Horan and Eccles (1986)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
High-speed centrifugation (20 000 – 48 000 x g)	Pure culture biofilm (*Klebsiella aerogenes*)	DNA + proteins	Brown and Lester (1980)
	Pure culture biofilm (*P. aeruginosa*)	G6PDH	Wingender *et al.* (2001)
	Various pure culture biofilms	n. d.	Buckmire (1984)
	Digested sewage sludge/activated sludge	n. d.	Pavoni *et al.* (1972)
		DNA	Liu and Fang (2002)
		DNA	Comte *et al.* (2006)
	Anaerobic granular sludge	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
	Freshwater/marine isolates	n. d.	Kennedy and Sutherland (1987)
Sonication	Pure culture biofilm (*K. aerogenes*)	Protein	Rudd *et al.* (1982)
		DNA + proteins	Brown and Lester (1980)
	Pure culture biofilm (*Sphingomonas paucimobilis*)	DNA	Azeredo *et al.* (1999)
	Pure culture biofilm (*P. fluorescens*)	ATP	Azeredo *et al.* (2003)
	Activated sludge	DNA + cell count	Jorand *et al.* (1995)
		n. d.	Quarmby and Forster (1995)
		n. d.	Urbain *et al.* (1993)
		n. d.	King and Forster (1990)
		n. d.	Dignac *et al.* (1998)
		DNA	Comte *et al.* (2006)

Table 1.2: *Continued*

Method	Sample	Indicator for cell lysis	Reference
		G6PDH	Ras *et al.* (2008)
		n. d.	Zhang *et al.* (2011)
	Anaerobic granular sludge	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
	Granular sludge	n. d.	Rahman *et al.* (1997)
Steam (Autoclaving)	Pure culture biofilm (*K. aerogenes, S. paucimobilis*)	DNA	Azeredo *et al.* (1999)
	Activated sludge	Protein/PS-ratio	Karapanagiotis et al (1989)
		n. d.	Hejzlar and Chudoba (1986)
		DNA + proteins	Brown and Lester (1980)
Ultracentrifugation (113 000 x g)	Pure culture biofilm (*Pseudomonas* sp.)	n. d.	Wrangstadh *et al.* (1986)
Ultra-Turrax	Pure culture biofilm (*R. capsulata*)	n. d.	Omar *et al.* (1983)
	Activated sludge	G6PDH	Ras *et al.* (2008)
Chemical isolation procedures (chemicals applied in combination with stirring)			
Crown-ether	Activated sludge	G6PDH	Wuertz *et al.* (2001)
	Benthic eukaryotic acidic biofilms	G6PDH	Aguilera *et al.* (2008)
Deionized water	Pure culture biofilm (*Sphaerotilus natans*)	n. d.	Gaudy and Wolfe (1962)
	Benthic eukaryotic acidic biofilms	G6PDH	Aguilera *et al.* (2008)
EDTA	Sediment bacterium	G6PDH + KDO	Platt *et al.* (1985)
	Activated sludge	n .d.	Nishikawa and Kuriyama (1968)
		DNA + proteins	Brown and Lester (1980)
		n. d.	Fang and Jia (1996)
		DNA	Liu and Fang (2002)
	Anaerobic sludge granules	DNA	Comte *et al.* (2006)
	Benthic eukaryotic acidic biofilms	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
	Pure culture biofilm (*R. acidophila*)	G6PDH	Aguilera *et al.* (2008)
	Pure culture biofilm (*Escherichia coli*)	DNA	Sheng et al (2005)
	Pure culture biofilm (*Shewanella* sp.)	DNA	Eboigbodin and Biggs (2008)
		cyclic AMP receptor protein	Cao *et al.* (2011)
	Pure culture biofilm (*Acinetobacter* sp.)	membrane integrity	Wu and Xi (2009)
Enzyme	Activated sludge	n .d.	Dey *et al.* (2006)
		Culturability	Sesay (2006)
		n. d.	Wawrzynczyk *et al.* (2007)
	Pure culture biofilm (*Acinetobacter sp.*)	G6PDH + membrane integrity	Wu and Xi (2009)

Table 1.2: *Continued*

Method	Sample	Indicator for cell lysis	Reference
Enzyme/NaOH	Pure culture biofilm (*Pseudomonas* sp.)	n. d.	Tago and Aida (1977)
Ethanol-precipitation	Activated sludge	n. d.	Forster and Clarke (1983)
	Paper machine slimes	n. d.	Rättö *et al.* (2006)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
Formaldehyde	Activated sludge	DNA	Liu and Fang (2002)
Formaldehyde/NaOH	Activated sludge	DNA	Liu and Fang (2002)
		DNA	Comte *et al.* (2006)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
Glutaraldehyde	Pure culture biofilm (*S. paucimobilis*)	DNA	Azeredo *et al.* (1999)
	Activated sludge	DNA	Comte *et al.* (2006)
H_2SO_4	Pure culture biofilm (*R. acidophila*)	DNA	Sheng *et al.* (2005)
K_2HPO_4	Pure culture biofilm (*S. paucimobilis*)	DNA	Azeredo *et al.* (1999)
	Pure culture biofilm (*Zoogloea*)	n. d.	Farrah and Unz (1976)
	Activated sludge	DNA	Azeredo *et al.* (1999)
NaCl	Pure culture biofilm (*P. putida, P. fluorescens*)	n. d.	Read and Costerton (1987)
	Pure culture biofilm (*P. aeruginosa*)	n. d.	May and Chakrabarty (1994)
	Pure culture biofilm (*Pseudomonas* sp. NCMB 2021)	n. d.	Christensen *et al.* (1985)
	Litoral sediments	n. d.	Underwood *et al.* (1995)
	Benthic eukaryotic acidic biofilms	G6PDH	Aguilera *et al.* (2008)
NaCl/EDTA	Pure culture biofilm (*Clostridium acetobutylicum*)	n. d.	Junelles *et al.* (1989)
NaOH	Pure culture biofilm (*E. coli*)	n. d.	Sato and Ose (1984)
	Pure culture biofilm (*R. acidophila*)	DNA	Sheng *et al.* (2005)
	Pure culture biofilm (*Acinetobacter* sp.)	Membrane integrity	Wu and Xi (2009)
	Activated sludge	n. d.	Sato and Ose (1984)
		DNA + proteins	Brown and Lester (1980)
		n. d.	Park and Novak (2007)
	Digested sewage sludge	Protein/PS-ratio	Karapanagiotis *et al.* (1989)
NH_4OH/EDTA	Activated sludge	n. d.	Sato and Ose (1984)
Phenol	Digested sewage sludge	Protein/PS-ratio	Karapanagiotis *et al.* (1989)
Pyridinacetat	Pure culture biofilm (*E. coli*)	n. d.	Pelkonen *et al.* (1988)
Sodium dodecyl sulphate	Pure culture biofilm (*Acinetobacter* sp.)	Membrane integrity	Wu and Xi (2009)
Sodium tripolyphosphate	Activated sludge	n. d.	Wawrzynczyk *et al.* (2007)
Sulfide	Activated sludge	n. d.	Nielsen and Keiding (1998)
		n. d.	Park and Novak (2007)
Tris/HCl	Pure culture biofilm (*S. paucimobilis*)	DNA	Azeredo *et al.* (1999)
Triton X-100	Activated sludge	G6PDH	Ras *et al.* (2008)

Table 1.2: *Continued*

Method	Sample	Indicator for cell lysis	Reference
Combinations of physical and chemical isolation methods			
Cetyltrimethylammoniumbromide/ heating	Pure culture biofilm (*R. capsulate*)	n. d.	Omar *et al.* (1983)
CER/stirring	Pure culture biofilm (*P. putida*)	G6PDH + culturability	Jahn and Nielsen (1995)
	Pure culture biofilm (*S. paucimobilis*)	DNA	Azeredo *et al.* (1999)
	Pure culture biofilm (*P. fluorescens*)	ATP	Azeredo *et al.* (2003)
	Pure culture biofilm (*Acinetobacter* sp.)	G6PDH + Membrane integrity	Wu and Xi (2009)
	Wastewater biofilm	G6PDH + culturability	Jahn and Nielsen (1995)
	Activated sludge	n. d.	Rudd *et al.* (1983)
		G6PDH	Frølund *et al.* (1996)
		DNA	Comte *et al.* (2006)
		Culturability	Sesay *et al.* (2006)
		n. d.	Park and Novak (2007)
		n. d.	Wawrzynczyk *et al.* (2007)
		G6PDH	Ras *et al.* (2008)
	Benthic eukaryotic acidic biofilms	G6PDH	Aguilera *et al.* (2008)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
	Microphytobenthic biofilm	n. d.	Pierre *et al.* (2012)
CER/formaldehyde	Activated sludge	n. d.	Rudd *et al.* (1983)
Ethanol/high-speed centrifugation	Activated sludge	DNA	Azeredo *et al.* (1999)
		G6PDH	Frølund *et al.* (1996)
Formaldehyde/heating	Activated sludge	n. d.	Fang and Jia (1996)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
Formaldehyde/sonication	Digested sewage sludge/activated sludge	n. d.	Fang and Jia (1996)
		DNA	Liu and Fang (2002)
Hexadecyltrimethylammonium-bromide/heating	Pure culture biofilm (*E. coli*)	n. d.	Schmidt and Jann (1982)
		n. d.	Jann *et al.* (1980)
NaCl/formaldehyde/sonication	Activated sludge	n. d.	Jia *et al.* (1996)
		n. d.	Fang and Jia (1996)
NaCl/heating	Pure culture biofilm (*R. capsulate*)	n. d.	Omar *et al.* (1983)
NaCl/sonication	Pure culture biofilm (*Staphylococcus epidermidis*)	n. d.	Evans *et al.* (1994)
NaOH/heating	Pure culture biofilm (*Rhizobacteria*)	n. d.	Hebbar *et al.* (1992)
	Pure culture biofilm (*Rhizobium trifolii*)	n. d.	Breeveld *et al.* (1990)
Phenol/sonication	Methanogenic granules	n. d.	Veiga *et al.* (1997)

Table 1.2: *Continued*

Method	Sample	Indicator for cell lysis	Reference
Sonication/Dowex	Activated sludge	n. d.	Dignac *et al.* (1998)
		DNA	Liu and Fang (2002)
		n. d.	Guibaud *et al.* (2003)
		DNA	Comte *et al.* (2006)
		G6PDH	Ras *et al.* (2008)
	Anaerobic sludge granules	DNA + protein/PS-ratio	D'Abzac *et al.* (2010)
Sonication/Triton X-100	Activated sludge	G6PDH	Ras *et al.* (2008)
Ultraturax/Dowex	Activated sludge	G6PDH	Ras *et al.* (2008)
Ultraturax/Triton X-100	Activated sludge	G6PDH	Ras *et al.* (2008)

- EPS quantification -

Colorimetric of fluorometric measurements are common methods to quantify EPS components (Tab. 1.3). The general principle of these assays is the reaction of the respective EPS component with chemicals to form products or complexes, which, in the case of photometric assays, absorb light of a certain wavelength, or in the case of fluorometric assays, are excited by light of a certain wavelength, while emitting light of a longer wavelength. These methods allow for a general estimation of the overall quantity of EPS components as sum parameter, relative to an appropriate standard. Furthermore, photometric or fluorometric assays are routinely used for the quantification of enzyme activities and can be adapted for total biofilm or EPS studies (Wingender and Jaeger, 2002). Examples of enzyme activities determined in the EPS of microbial biofilms are given in Tab. 1.1.

Table 1.3: Photometric or fluorometric assays used for the quantification of EPS components.

EPS component	Main reagent(s)	Reference
Proteins	Alkaline copper, Folin-Ciocalteu reagent	Lowry *et al.* (1951)
	Alkaline copper / SDS, Folin-Ciocalteu reagent	Peterson (1977)
	Bicinchoninic acid	Smith *et al.* (1985)
	Coomassie Brilliant Blue G-250	Bradford (1976)
	Ninhydrin	Rosen (1957)
Carbohydrates	H_2SO_4 / phenol	Dubois *et al.* (1956)
	Anthrone	Morse (1947)
Uronic acids	m-Hydroxydiphenyl	Blumenkrantz and Asboe-Hansen (1973) Filisetti-Cozzi and Carpita (1991)
DNA	DAPI	Brunk *et al.* (1979)
	Diphenylamine	Burton (1956)
	PicoGreen	Ahn *et al.* (1996) Singer *et al.* (1997)
Lipids	H_2SO_4 / phospho-vanillin	Frings *et al.* (1972)
Lipopolysaccharides	H_2SO_4, HIO_4, $NaAsO_2$, thiobarbituric acid	Karkhanis *et al.* (1978)
Humic acids	Modified Lowry assay	Frølund *et al.* (1995)

- Electrophoretic and chromatographic EPS analysis -

Qualitative analysis of EPS components requires additional clean-up and separation steps of the respective group of polymers to allow for their characterization. Electrophoretic or chromatographic approaches, such as one- or two-dimensional gel electrophoresis, denaturing gradient gel electrophoresis, thin-layer chromatography (TLC), gas chromatography (GC) or high performance liquid chromatography (HPLC), have been developed or adapted in order to analyse the biofilm matrix.

Qualitative analysis of proteins is usually performed by one-dimensional or two-dimensional gel electrophoresis (1DE and 2DE, respectively). 1DE is a rapid method to separate proteins by size on a polyacrylamide gel and is adequate to obtain band-patterns for protein samples. This method, however, is limited to samples with a low diversity of proteins. Complex samples require more sophisticated methods to achieve a thorough separation of the entire proteome. The development of 2DE, as it is applied nowadays, provides a by far more sensitive technique to study protein samples. 2DE separates proteins in the first dimension according to their charge by isoelectric focusing (IEF), before they are further separated by molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. This allows for separation of over 5000 proteins on a single gel and provides a detailed fingerprint of the sample's proteome (O'Ferrell, 1975; Görg *et al.*, 2004). Subsequent spot analysis for example by matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) or electrospray ionisation mass spectrometry (ESI-MS) allows identification of the proteins (Denkhaus *et al.*, 2007). A weakness of this method, however, is the low throughput of samples, the reproducibility due to gel-to-gel variations, the limited amount of mass spectra in databases and potential interferences of non-proteinaceous compounds with the IEF step. This is especially of significance for the analysis of extracellular proteins. Studies identifying EPS proteins are very scarcely represented in literature and up to date EPS proteins from drinking-water biofilms have not been described qualitatively. Furthermore, the EPS matrix can contains substances, which interfere with IEF (Rémy *et al.*, 2000; Görg *et al.*, 2004). IEF is the most delicate step during 2DE, being very sensitive to impurities within the protein sample. Broekman (2009) described the immensely detrimental effect of alginate from *P. aeruginosa* on IEF as well as on conventionally applied methods for protein purification for IEF, and eliminated alginate by ultrafiltration. Also

nucleic acids or high salt contents are known to interfere with the IEF by clogging pores of the gel or providing a high current during IEF, respectively (Rémy *et al.*, 2000; Görg *et al.*, 2004). DNase treatment to digest interfering DNA and subsequent dialysis to reduce salt content and to remove low molecular weight organic substances are means for protein clean-up for IEF. 2DE is not only applied to identify the proteome of a culture, it is in particular applied to compare protein expression under different cultivation conditions or environmental stress. In this case, however, a large number of technical and biological replicates needs to be performed to be able to exclude gel-to-gel variations or variations within the same samples. In the attempt to overcome this problem, advancements resulted in the recent development of the 2D difference gel electrophoresis (2D-DIGE) (Ünlü *et al.*, 1997; Tonge *et al.*, 2001). This method allows for comparison of a set of proteomes, by separation of proteins from two different samples on a single gel, eliminating the need of technical replicates. 2D-DIGE software not only detects the presence or absence of proteins, it is also capable of quantifying up or down regulation of protein expression in the respective set of samples.

Chromatographic approaches, such as ion exchange or size exclusion chromatography, are commonly applied for the separation and purification of proteins from undesired contaminants, for instance polysaccharides or nucleic acids, but also for concentration of the protein of choice. Chromatography has, furthermore, been described as potent tool for the identification of proteins from microbial biofilms. Ram *et al.* (2005) used an HPLC approach in the form of 2D nano-liquid chromatography tandem mass spectrometry to identify proteins in a biofilm community from natural acid mine drainage. Recent developments of labelling techniques, such as Isotope Coded Affinity Tags (ICAT) (Gygi *et al.*, 1999; Han *et al.*, 2001), iTRAQ (Ross *et al.*, 2004; Zieske, 2006), or aniline benzoic acid labelling (ANIBAL) (Panchaud *et al.*, 2008) in combination with HPLC and mass spectrometry, allow for simultaneous qualitative analysis as well as assessment of the relative quantities of proteins within multiplexed samples. Application of these techniques for complex environmental samples, however, has not yet been reported, but should be considered as promising alternatives for the analysis of biofilm proteomes (Schneider and Riedel, 2010).

Polysaccharide analysis usually involves chromatographic methods to characterize polysaccharides by their molecular weight or in terms of their monosaccharide composition.

Size exclusion chromatography is widely applied to separate polysaccharides according to their molecular weight (Gaborieau and Castignolles, 2011). Modifications of this type of chromatography, i.e. size-exclusion capillary electrochromatographic separation, allows for separation of polysaccharides as large as 112,000 g mol^{-1} (Mistry *et al.*, 2003). Identification of the qualitative monosaccharide composition of polysaccharides requires hydrolysis of the polymer into its monomers. The most common hydrolytic agents applied are acids, such as hydrochloric acid, sulphuric acid or trifluoroacetic acid, which degrade polysaccharides at high temperatures into its components (Denkhaus *et al.*, 2007). Obtained mono- or oligosaccharides can then be separated by chromatography. Identification of the exopolysaccharide composition has for example been performed by use of TLC of exopolysaccharides from *Bacillus thermoantarcticus* biofilms (Manca *et al.*, 1996) or *P. aeruginosa* biofilms (Rode, 2004). Dignac *et al.* (1998) described the sugar composition of activated sludge floc polysaccharides applying GC (Dignac *et al.*, 1998). Meisen *et al.* (2008) presented an HPLC in combination with refractive index/UV detection technique for the analysis of alginate purified from a *P. aeruginosa* biofilm.

- *Spectroscopic methods* -

A number of spectroscopic methods have been adapted for use in biofilm research. These include Fourier-transformed infrared (FT-IR) or Raman spectroscopy, which give information on fuctional groups, structural features, conformational changes or adsorption reactions within the biofilm matrix with spatial resolution in the micrometer range (Suci *et al.*, 2001; Ivleva *et al.*, 2010; Sheng *et al.*, 2010). These spectroscopic methods allow for investigations of fully hydrated samples with minimal sample preparation, thus minimizing artifacts caused by sample manipulation (Denkhaus *et al.*, 2007). FT-IR in the form of attenuated total reflection (ATR/FT-IR) has been applied in a number of biofilm studies, for example to analyse adhesion and growth of biofilms under varying conditions (Nivens *et al.*, 1993; Schmitt *et al.*, 1995), to investigate the penetration of the antibiotic ciprofloxacin and its interaction with EPS components (Suci *et al.*, 1994) or to assess conformational changes of EPS proteins in response to variations in solution chemistry (Omoike and Chorover, 2004). Raman spectroscopy has been applied for example for comparative studies of *S. epidermidis*

wild-type and mutant biofilms (Samek *et al.*, 2010), as well as *P. fluorescens* biofilm cells and planktonic cells (Huang *et al.*, 2007). A few studies applied combinations of both methods for an in-depth analysis of biofilms. Choo-Smith *et al.* (2001) used ATR/FT-IR as well as Raman spectroscopy to investigate microbial colony heterogeneity of different bacterial strains, while Suci *et al.* (2001) analyzed chlorhexidine spatial and temporal distribution in *Candida albicans* biofilms. Recently the Raman spectrometry-based method surface-enhanced Raman scattering (SERS) has been developed and applied for the analysis of biofilms. Ivleva *et al.* (2010) demonstrated SERS' applicability for *in situ* analysis of multi-species biofilms, biofilm constituents and their spatial distribution.

Nuclear magnetic resonance (NMR) spectroscopy represents a technique capable of providing monomeric (Mayer *et al.*, 2001; Schürks *et al.*, 2002), or structural information of isolated polysaccharides (Gruter *et al.*, 1993; Jann *et al.*, 1994) or proteins (Wüthrich, 2001), interactions of EPS components with metals (Lattner *et al.*, 2003) or monitoring metabolic turnover in cell suspensions (Brecker and Ribbons, 2000). The application of NMR for *in situ* biofilm research, however, has been limited to investigations of water properties (Majors *et al.*, 2005; Denkhaus *et al.*, 2007). These studies included determinations of the hydrodynamic properties of water at biofilm surfaces (Lewandowski *et al.*, 1992, 1995; Manz *et al.*, 2003; Seymour *et al.*, 2004), as well as water diffusion in the EPS matrix (Vogt *et al.*, 2000).

- *Mineral composition* -

Multivalent cations like Ca^{2+} or Mg^{2+} are known to play a major role for the mechanical stability of biofilms (Körstgens *et al.*, 2001; Wloka *et al.*, 2004). Furthermore, mechanisms such as biosorption, precipitation as sulfides or phosphates and microbial reductive precipitation are involved in the immobilisation and accumulation of heavy metals within biofilms (van Hullebusch *et al.*, 2003). Despite their profound influence on biofilm integrity and composition, inorganic substances are often disregarded in biofilm and EPS investigations (D'Abzac *et al.*, 2010). Atomic spectrometry, such as atomic absorption spectrometry (AAS), or inductively coupled plasma coupled with optical emission spectrometry (ICP-OES) or mass spectrometry (ICP-MS) are routine methods used for the

quantification of metals in liquid samples. A few studies have adapted these methods to investigate metal sorption properties of biofilms. Pretreatment of the biomass by acid digestion at high temperatures (White and Gadd, 1996, 1998, 2000), or in combination with microwave treatment (Farag *et al.*, 2007; D'Abzac *et al.*, 2010) has been implemented and allows for application of the samples for AAS or ICP analyses. Toxic metals like copper, zinc, cadmium or lead were common metals of interest in studies of sulphate-reducing mixed biofilms, wastewater or activated sludge biofilms (Späth *et al.*, 1998; White and Gadd, 1998, 2000; Comte *et al.*, 2008; Guibaud *et al.*, 2009). Park and Novak (2007) determined the concentrations of Ca, Mg, Fe and Al in activated sludge and evaluated the efficiency of the CER Dowex as well as its selectivity towards certain cations, based on the removal of these metals during EPS isolation. D'Abzac *et al.* (2010) applied ICP-OES and ICP-MS for a multi-element analysis of anaerobic granular sludges. Their study provides a broad-range quantification of the mineral fraction of the EPS, including Ca, K, Mg, Na, P, S, Si, Al, Fe, Mn, Ni, Cu, As, Ag, Cd and Pb by ICP techniques (D'Abzac *et al.*, 2010).

1.4.2 Tools to study biofilm architecture

Diverse microscopic methods have been designed to give an insight into the architecture of biofilms. Light or epifluorescence microscopy can give a first indication of the structure of microbial slime, however, more sophisticated methods, such as those described below, as well as combinations of different microscopic methods are required to elucidate the complex heterogeneity and spatial distribution of cells, polymers or minerals in fully hydrated biofilm samples.

- Electron microscopy -

Electron microscopy has been developed in the 1930s and provides powerful techniques achieving a resolution higher than 50 pm (Erni *et al.*, 2009). These techniques apply an electron beam, which interacts with the sample to produce an image. The most widely applied methods in biofilm research include scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Their applicability for biofilm research has been

shown in different studies, for example investigating anaerobic fixed film fermenter biofilms (Richards and Turner, 1984), natural aquatic biofilms (Surman *et al.*, 1996), microbially influenced corrosion (Steele *et al.*, 1994; Chen *et al.*, 1997) or *P. aeruginosa* biofilms (Priester *et al.*, 2007). The high resolution power of these methods allows not only for visualization of microbial cells, also cell organelles, EPS components and minerals can be visualized. Coupling of SEM with an energy dispersive x-ray analysis (EDX) generates images and quantifies relative concentrations of minerals within the observed sample region (Chen *et al.*, 1997; D'Abzac *et al.*, 2010). Despite the high resolution power these techniques have a flaw. Sample preparation includes chemical fixation and/or drying, which causes a breakdown of the original biofilm structure and produces artifacts (Richards and Turner, 1984). Advancements of these microscopic methods, such as the development of a gaseous secondary electron detector device for environmental SEM (ESEM), allow microscopic analysis of fully hydrated samples (Danilatos, 1981, 1983). Theoretically, samples no longer have to be fixed or dried for ESEM, however, resolution is lower compared to SEM and by the time samples are loaded and observed, drying occurs and alters biofilm structure.

- *Confocal laser scanning microscopy* -

Confocal laser scanning microscopy (CLSM) is the by far most applied tool to study the three-dimensional structure of biofilms and its components. This method allows for investigations of a multitude of features in a fully hydrated, multi-layered biofilm sample (Lawrence and Neu, 2003). CLSM can be performed in two modes, the rarely applied reflection mode, which can give information about minerals, colloids, cell surfaces or cell inclusions (Lawrence and Neu, 2003), or the fluorescence mode, which relies on autofluorescence of the sample or fluorescent labelling of the sample component of interest. In the latter case, the fluorophore is excited by a laser of a specific wavelength, while emitted light of the corresponding wavelength is detected, generating an image. The application range covers investigations of the autofluorescence of phototrophic algae or cyanobacteria, visualization of the spatial distribution of cells or EPS components, identification of microorganisms of interest, viability and cell activity, localization of enzyme activity, as well as diffusion, permeability, pH, ions or metal studies (Lawrence and Neu, 2003). Multi-channel CLSM analysis allows for

simultaneous investigation of several of these parameters. To be able to investigate these different aspects of biofilms, a large number of stains have been developed, aiming at selective binding to nucleic acids, polysaccharides or proteins, gene-markers, for selective visualization of microorganisms of interest, or fluorescent substrates specific for different classes of enzymes (Tab. 1.4). Due to their high specificity towards certain carbohydrate binding sites (Kennedy, 1995), fluor-conjugated lectins have been of particular use for *in situ* determination of the spatial distribution of polysaccharides in biofilms by CLSM (Neu *et al.*, 2001, 2005; Strathmann *et al.*, 2002). The specificity of each stain needs to be considered, as studies have shown, that the biofilm matrix can inhibit or enhance binding of the fluorophores, in particular lectins, to the respective target (Neu *et al.*, 2001).

Table 1.4: Fluorescent stains or markers applied for microscopical analysis of biofilms (adapted and supplemented from Strathmann, 2002).

Target	Stain or marker	Reference
Polysaccharides, Glycoconjugates	Alcian blue	Wetzel *et al.*, 1997; Karlyshev and Wren, 2001
	Calcofluor white	Wood, 1980; von Sengbusch *et al.*, 1982; Marshall, 1992; Ramaswamy *et al.*, 1997; Chandra *et al.*, 2001; Kuhn *et al.*, 2002
	Congo red	Wood, 1980; Allison und Sutherland, 1984; Marshall, 1992; Lawrence *et al.*, 1998a
	Ruthenium red	Gutierrez-Gonzalvez *et al.*, 1987
	Fluorescently-labelled lectins	Sizemore *et al.*, 1990; Caldwell *et al.*, 1992; Quintero and Weiner, 1995, Hood and Schmidt, 1996; Holloway and Cowen, 1997; Neu and Lawrence, 1997, 1999; Langille and Weiner, 1998; Lawrence *et al.*, 1998a; Johnsen *et al.*, 2000; Neu *et al.*, 2001; Wingender *et al.*, 2001; Strathmann *et al.*, 2002
	Peroxidase-marked lectins	Leriche *et al.*, 2000; Strathmann *et al.*, 2002
	Biotinylised lectins	Sanford *et al.*, 1995; Kawaguchi and Decho, 2000
	Antibodies	Costerton *et al.*, 1981; Weiner *et al.*, 1995
Proteins	Hoechst 2495	Paul and Jeffrey, 1985; Neu and Marshall, 1991
	Nano orange	Grossart *et al.*, 2000
	Sypro Red	Strathmann, 2002
	Sypro Ruby	Strathmann, 2002; Di Poto *et al.*, 2009
	Antibodies	Dazzo and Wright, 1996

Table 1.4: *Continued*

Target	Stain or marker	Reference
Nucleic acids	Acridin orange	Wentland *et al.*, 1996; Mattila *et al.*, 1997; Van Ommen Kloeke and Geesey, 1999
	DAPI	Huang *et al.*, 1995; Van Ommen Kloeke and Geesey, 1999; Espeland and Wetzel, 2001
	DDAO	Allesen-Holm *et al.*, 2006; Dominiak *et al.*, 2011
	Propidium iodide	Van Ommen Kloeke and Geesey, 1999; Davey and O'Toole, 2000
	Syto dyes	Mattila *et al.*, 1997; Van Ommen Kloeke and Geesey, 1999; Davey and O'Toole, 2000; Strathmann *et al.*, 2002
	Gene probes	Wagner *et al.*, 1994; Amann *et al.*, 1995; Wagner and Amann, 1997; Manz *et al.*, 1999; Böckelmann *et al.*, 2000; Davey and O'Toole, 2000; Nielsen *et al.*, 2000
Lipids	Nil red	Lamont *et al.*, 1987
Enzyme activity	CTC	Rodriguez *et al.*, 1992; Schaule *et al.*, 1993; Walsh *et al.*, 1995; Wuertz *et al.*, 1998
	ELF-97 substrates	Huang *et al.*, 1993; Huang *et al.*, 1998; Van Ommen Kloeke and Geesey, 1999; Baty III *et al.*, 2000, 2001
	INT	Chung and Neethling, 1989; Kim *et al.*, 1994; Walsh *et al.*, 1995
	TTC	Ryssov-Nielsen, 1975; Miksch, 1985
Hydrophobic regions	Nile red	Lamont *et al.*, 1987; Wolfaardt *et al.*, 1994, 1998
	Hydrophobic, fluorescent micro-beads	Zita und Hermansson, 1997
pH	Carboxyfluorescein	Vroom *et al.*, 1999
Gram-Stain	BacLight Gram stain	Wolfaardt *et al.*, 1998 ; Karthikeyan *et al.*, 1999
Permeability	Fluorescent dextrans	Korber *et al.*, 1994; Lawrence *et al.*, 1994; Birmingham *et al.*, 1995; Wolfaardt *et al.*, 1998
	Fluorescent micro-beads	Stoodley *et al.*, 1994; Lawrence *et al.*, 1998b

- *Scanning probe microscopy* -

Atomic force microscopy (AFM) is among the most commonly applied scanning probe technique (Denkhaus *et al.*, 2007). AFM uses a cantilever, which is dragged or tapped along the biofilm surfaces to produce a highly resolved topographic image of the sample at the atomic scale. This technique is capable of visualizing single biopolymers, for instance DNA

(Allison *et al.*, 1997), proteins (Chen *et al.*, 1998) or DNA-protein interactions (Rippe *et al.*, 1997). This type of microscopy has also been applied for visualization of microbial EPS. Steele *et al.* (1994) investigated microbially influenced corrosion of stainless steel by biofilm organisms by AFM. Beech (1996) and Beech *et al.* (2002) used AFM for investigations of microbial biofilms formed by sulfate-reducing bacterial biofilms on steel surfaces and their interactions with the substratum. Van der Aa and Dufrêne (2002) performed an *in situ* characterization of the organization of EPS produced by *Azospirillum brasilense* attached to polystyrene substrata by application of AFM. Li and Logan (2004) used an AFM approach to determine the adhesion of different *E. coli* strains to be able to predict microbial adhesion. However, the AFM method is mainly suitable for investigations of bacterial monolayers. Scanning probe microscopy furthermore includes techniques like scanning tunneling electron microscopy and scanning ion-conductance microscopy (Hansma and Pietrasanta, 1998).

1.5 Aims of the study

Drinking water distribution systems as well as domestic plumbing systems are colonized by microbial biofilms, which under unfavourable conditions can act as reservoirs for pathogenic microorganisms, posing a potential threat to human health (Bressler *et al.*, 2009; Moritz *et al.*, 2010; Wingender, 2011). Despite numerous studies investigating biofilm formation in these systems, none has addressed the EPS composition of drinking-water biofilms. To be able to understand, control and avoid biofilm formation, it is essential to gain an understanding of the involved microorganisms as well as the quantity and composition of EPS, both of which are targets for measures aimed at the inactivation and removal of biofilm organisms from these systems.

This study was carried out to characterize drinking-water biofilms and their EPS grown in drinking water distribution systems as well as public plumbing systems by microbiological, molecular biological and biochemical means. The aims of this study included:

- Adaptation and optimization of analytical methods for the analysis of the typically low amounts of drinking-water biofilms found in drinking water systems, as well as their EPS, which have not been analyzed in drinking-water biofilms in detail before,
- Evaluation of EPS isolation methods based on their EPS yield, detrimental impact on biofilm cells, as well as compatibility with subsequent analytical methods, with main focus on CER isolation,
- Application of the established methods for the cultivation and characterization of drinking-water biofilms cultivated in reactors connected to plumbing systems of public buildings or to drinking water distribution systems,
- Qualitative EPS analysis of drinking-water biofilms, with focus on the identification of extracellular proteins, which unlike exopolysaccharides have been rarely investigated in literature, applying two-dimensional gel electrophoresis, as well as enzyme activity measurements, and
- Investigation of the incorporation of the hygienically relevant *P. aeruginosa* in drinking-water biofilms and its influence on biofilm community and EPS composition.

2. MATERIALS

2.1 Chemicals

Table 2.1: Chemicals used in this study.

Substance	Specification	Manufacturer	Art. No.
Acetic acid	AnalaR 100 % Normapur	VWR	20104.334
Acetone	Analytical reagent grade	Fisher Chemicals	A/066/17
Acetonitrile	Chromasolve gradient grade	Sigma-Aldrich	34851-2.5L
Acrylamide/Bis solution (40 %)	37.5:1	Bio-Rad	161-0148
Agarose	Research grade	Merck	1.16801.0250
L-alanine-4-methoxy-2-naphthylamide		Sigma	A1541
Ammonium hydrogencarbonate	Ultra > 99.5 %	Fluka BioChemika	09830
Ammonium persulfate	≥ 98 %, p.a., ACS	Roth	9592.2
Bovine serum albumin standard		Sigma	P5619
Bovine albumin	≥ 96 %	Sigma	A4503
Bromphenol blue indicator	pH 3.0 – 4.6	Merck	3627503
2-Butanol	p. a.	Merck	1.09630.1000
[3-(3-Cholanamido-propyl)dimethylammonio]-1-propanesulfonate (CHAPS)		Calbiochem	220201
Coomassie Brilliant blue G250	For electrophoresis	Merck	1.15444.0025
α-Cyano-4-hydroxycinnamic acid	p. a.	Fluka	70990-1G-F

Table 2.1: *Continued*

Substance	Specification	Manufacturer	Art. No.
Dextran	From *Leuconostoc mesenteroides*	Sigma	D5376
4,'6-Diamidino-2-phenylindole dihydrochloride (DAPI)	≥ 98 %	Sigma	D9542
DL-Dithiothreitol		Sigma	43815
Dowex	Marathon C, Na^+-form, strongly acidic, particle size 20 – 50 mesh	Sigma	91973
Ethanol	Rotipuran	Roth	9065.4
Ethylenediaminetetraacetic acid tetrasodium salt (Na_4EDTA)		Sigma	ED4SS
Folin-Ciocalteu's phenol reagent	2 mol/L	Sigma	F9252
Formaldehyde solution	~ 36 % in H_2O	Fluka	47630
Formamide deionized	≥ 99.5 % p.a.	Roth	P040.1
Formamide	For molecular biology	Sigma	47671
Glass beads	≤ 106 µm	Sigma	G4649
D-Glucose 6-phosphate monosodium salt	98.5 %	Sigma	F7879
D-Glucose	99.5 %	Sigma	F7528
D-Glucuronic acid		Sigma	F5269
Glycerin	ACS	Merck	1.04092.1000
m-Hydroxybiphenyl	90 %	Sigma	H6527
Iodoacetamide		Sigma	I6125
2-keto-3-deoxyoctonate ammonium salt		Sigma	K2755
Loading Dye TriTrack	6x concentrate	Fermentas	R1161
Lowry reagent modified		Sigma	L3540
Magnesium chloride hexahydrate	p. a.	Merck	1.05833.0250
Mark12 unstained standard		Invitrogen	LC5677
MassRuler DNA Ladder mix	103 µg $µL^{-1}$ DNA	Fermentas	SM0403
Methanol	Analytical reagent grade	Fisher Chemical	M/4000/17
2-methoxy-ethanol	p. a. ACS	Roth	CP08.2
4-methoxy-2-naphthylamine		Sigma	M9894

Table 2.1: *Continued*

Substance	Specification	Manufacturer	Art. No.
4-methylumbelliferone (MUF)		Sigma	M1381
4-MUF-α-D-glucopyranoside		Sigma	M9766
4-MUF-ß-D-glucopyranoside		Sigma	M3633
4-MUF-N-acetyl-ß-D-glucosaminide		Sigma	M2133
4-MUF-stearat		Sigma	M1010
4-MUF-butyrate		Sigma	19362
4-MUF-phosphate		Sigma	M8883
Nicotine amide adenine dinucleotide phosphate sodium salt		Sigma	N0505
Paraformaldehyde		Merck	1.04005
Phenol	≥ 99.5 %	Riedel-de Haën	33517
Potassium hexacyanoferrate (III)	p. a.	Merck	1.04973.0100
Potassium hydroxide		Baker	0385
Protease inhibitor cocktail	AEBSF, 23 mM; Bestatin, 2 mM; EDTA, 100 mM; E-64, 0.3 mM; Pepstatin A, 0.3 mM	Sigma	P8465
Roti-Load 2 sample buffer	Non reducing, 4x	Roth	K930.1
Rotipuran Water	p. a. ACS	Roth	T172.2
Servalyt Ampholyte	pH 3 – 10	Serva	42940
Silver nitrate	GR for analysis	Merck	1.01512
Sodium dodecyl sulfate (SDS)	≥ 98.5 %, for molecular biology	Sigma	L4390
Sodium hydroxide pellets	p. a.	KMF	08-620.1000
Di-sodium tetraborate-decahydrate	≥ 99.5 %	Fluka	72000
Sodium thiosulfate pentahydrate	p.a. ACS	Merck	1.06516
Sulfamic acid	≥ 99 %	Sigma	242772
Sulfuric acid	≥ 95 %	Fisher Scientific	S/9240/PB17
N,N,N',N'-Tetramethylethylenediamine (TEMED)	p. a.	Bio-Rad	161-0800

Table 2.1: *Continued*

Substance	Specification	Manufacturer	Art. No.
Thiourea	GR for analysis	Merck	1.07979
Tributyl phosphine	~ 95 %	Fluka	90827
Trifluoroacetic acid	For protein sequence analysis	Merck	1.08178.0050
Tris, Tris-(hydroxmethyl)-aminomethane	≥ 99.9 %	Roth	4855.1
Urea, Carbamide	≥ 99.5 %, BioUltra, for molecular biology	Sigma	51456
Water	Ultrapur	Merck	1.01262.100
Water for molecular biology	DEPC treated	Roth	T143.2

2.2 Cultivation media

BCYEα ready to use agar plates (OXOID):

Composition in g L^{-1} (ISO 11731:1998 (E)): Yeast extract 10.0, agar 12.0, activated charcoal 2.0, α-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide (KOH) 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25.

LB medium (Lennox Broth):

Composition in g L^{-1}: Tryptone 10.0, NaCl 5.0, yeast extract 5.0, pH 7.0 ± 0.2 at 25 °C.

Preparation: The compounds were dissolved in deionized water, pH was adjusted to 7.0 ± 0.2 and filled with deionized water to 1 L.

Nutrient agar (Merck):

Composition in g L^{-1}: Peptone from meat 5.0, meat extract 3.0, agar 12.0, pH 7.0 ± 0.2 at 25 °C.

Preparation: 20 g of the commercially available granulate were dissolved in 1 L of deionized water. 25 ml aliquots were poured into sterile Petri dishes.

Pseudomonas selective agar with C-N supplement (CN agar) (Oxoid):

Composition in g L^{-1}: Gelatin peptone 16.0, casein hydrolysate 10.0, potassium sulphate 10.0, $MgCl_2$ 1.4, agar 11.0, pH 7.1 ± 0.2 at 25 °C.

Preparation: 24.2 g of the granulate and 5 mL glycerol were dissolved in 500 mL of deionized water and autoclaved at 121 °C for 20 min. The content of one vial Pseudomonas C-N supplement (100 mg cetrimide, 7.5 mg sodium nalidixate; Oxoid) was dissolved in a 1:1 (v/v) solution of ethanol and sterile deionized water and aseptically added to the autoclaved agar base once it has cooled to 50 °C. 25 ml aliquots were poured into sterile Petri dishes.

R2A medium (Difco):

Composition in g L^{-1}: Yeast extract 0.5, proteose peptone No. 3 0.5, casamino acids 0.5, dextrose 0.5, soluble starch 0.5, sodium pyruvate 0.3, dipotassium phosphate 0.3, magnesium sulphate 0.05, agar 15.0, pH 7.2 ± 0.2 at 25 °C.

Preparation: 18.2 g R2A medium were dissolved in 1 L deionized water and autoclaved at 121°C for 20 min. 25 ml aliquots were poured into sterile Petri dishes.

2.3 Buffers and solutions

Agarose solution, 1 %

1 g agarose was suspended in 2 mL 50 x TAE-Puffer and 98 mL deionized water and dissolved by heating. The agarose solution was stored at 55 °C until use.

Ammonium persulphate (APS) solution, 10 %

0.1 g APS were dissolved in 1 mL deionized water.

Ammonium persulphate (APS) solution, 40 %

0.4 g APS were dissolved in 1 mL deionized water.

Benzonase buffer (pH 8.0), 10 x

6.057 g Tris (500 mM) and 0.203 g $MgCl_2$ x 6 H_2O (10 mM) were dissolved and filled up to 100 mL in deionized water and adjusted to pH 8.0. The buffer was filter sterilized (cellulose acetate filter, pore size 0.2 µm).

DAPI stain solution (25 µg mL^{-1}) in 2 % (v/v) formaldehyde

12.5 mg 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were dissolved in 27 mL formaldehyde (37 %) and 473 mL deionized water and filter sterilized (cellulose acetate filter, pore size 0.2 µm).

0 % denaturant solution in 7.5 % acrylamide

18.8 mL of a 40 % acrylamid solution were mixed with 2 mL 50 x TAE-Puffer and 79.2 mL Rotipuran water.

100 % denaturant solution in 7.5 % acrylamide

18.8 mL of a 40 % acrylamid solution were mixed with 2 mL 50 x TAE-Puffer, 40 mL formamide, 42 g urea and Rotipuran water. The solution was filled up to 100 mL with Rotipuran water once all ingredients were dissolved and the solution reached room temperature.

EDTA solution, 0.25 M

10.45 g Na_4EDTA x 2 H_2O were dissolved and filled up to 100 mL in Rotipuran water and autoclaved for 20 min at 121 °C.

EDTA solution, 2 %

2.848 g Na_4EDTA x 2 H_2O were dissolved and filled up to 100 mL with deionized water and autoclaved for 20 min at 121 °C.

Equilibration buffer for 2DE

72 g urea (6 M), 60 g glycerine (30 %), 4 g SDS (2 %) and 6.6 mL 1.5 M Tris/HCl-buffer (pH 8.8) were dissolved in deionized water and filled up to 200 mL with deionized water, once the solution had reached room temperature.

Ethanol-PBS

Ethanol for molecular biology was mixed with PBS in a ratio of 1:1 (v/v).

Hybridization buffer for Psae 16S-182 probes

For one 8-well diagnostic slide 1 mL hybridization buffer was prepared by mixing 400 µL Rotipuran water, 180 µL 5 M NaCl, 20 µL 1 M Tris (pH 8.0), 1 µL 10 % SDS and 400 µL formamide.

IEF buffer for 2DE

21.02 g urea (7 M), 7.61 g thiourea (2 M), 2 g CHAPS (4 %), 0.0625 mL tributylphosphine (5 mM) and 0.3125 mL Servalyte 3-10 ampholytes (m/v) (0.25 %) were dissolved and filled up to 50 mL with ultrapure water.

Psae 16S-182 oligonucleotide probe solutions

The *P. aeruginosa* specific, Cy3-labelled (5' end) oligonucleotide probe Psae 16S 182 (5'-CCA CTT TCT CCC TCA GGA CG- 3'; Wellinghausen *et al.*, 2005) was obtained as lyophilisate from Eurofins MWG Operon (Ebersbach, Germany). The lyophilisate was dissolved in water for molecular biology to a final concentration of 1 µg μL^{-1}. The dissolved lyophilisate was further diluted in water for molecular biology to obtain stock solutions of 50 µg mL^{-1}, which were stored as 20 µL aliquots at -20 °C until use. Working solutions of the oligonucleotide probe were prepared immediately prior to use by diluting the stock solution 10 fold in hybridization buffer.

Paraformaldehyde in PBS, 4 %

4 g paraformaldehyde were dissolved in PBS by stirring at 50 °C. The solution was cooled to room temperature and filled up to 100 mL in PBS. 4 mL aliquots were stored at -20 °C.

Phosphate buffer (pH 7.0), 6 mM

0.76 g Na_3PO_4 x 12 H_2O (2 mM), 0.552 g NaH_2PO_4 x 1 H_2O (4 mM), 0.526 g NaCl (9 mM) and 0.075 g KCl (1 mM) were dissolved and filled up to 1 L with deionized water and adjusted to pH 7. The buffer was autoclaved for 20 min at 121 °C.

Phosphate-buffered saline (PBS, pH 7.2)

8.0 g NaCl, 0.2 g KCl, 1.81 g Na_2HPO_4 x 2 H_2O and 0.24 g KH_2PO_4 were dissolved in Rotipuran water and adjusted to pH 7.2 and the solution was autoclaved for 20 min at 121 °C.

Sodium chloride solution, 5 M

29.2 g NaCl were dissolved and filled up to 100 mL with Rotipuran water and autoclaved for 20 min at 121 °C.

SDS solution, 10 %

10 g SDS were filled up to 100 mL with deionized water.

SDS-Tris-glycine running buffer, 1 x

150 mL of the commercially available Rotiphorese 10 x SDS-PAGE buffer (Roth) were filled up to 1.5 L with deionized water. Final composition: 0.192 M glycine, 0.025 M Tris, 0.1 % (w/v) SDS.

Sodium hydroxide solution, 1 M

4 g NaOH pellets were dissolved and filled up to 100 mL with deionized water and autoclaved for 20 min at 121 °C.

TAE buffer, 1 x

40 mL of the commercially available 50 x TAE buffer (Bio-Rad) concentrate were filled up to 1 L with deionized water. Final composition according to the manufacturer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3.

Tris/HCl-buffer (pH 8.0), 1 M

12.1 g Tris(hydroxymethyl)-aminomethane were dissolved in Rotipuran water. The pH value was adjusted to 8.0. The solution was filled up to 100 mL with Rotipuran water and autoclaved for 20 min at 121 °C.

Tris/HCl-buffer (pH 8.8), 1.5 M

90.855 g Tris(hydroxymethyl)-aminomethane were dissolved in deionized water. The pH value was adjusted to 8.8 and the solution was filled up to 500 mL with deionized water.

Washing buffer for Psae 16S-182 probes

For one 8-well diagnostic slides 25 mL washing buffer were prepared. 500 µL 1 M Tris/HCl-buffer (pH 8.0), 280 µL 5 M NaCl, 500 µL 0.25 M EDTA and 25 µL 10 % SDS were filled up to 25 mL with Rotipuran water.

Zymogram developing buffer, 1 x

10 mL of the commercially available zymogram developing buffer (10 x) (Invotrogen) concentrate were filled up to 100 mL with deionized water.

Zymogram renaturing buffer, 1 x

10 mL of the commercially available zymogram renaturing buffer (10 x) (Invitrogen) concentrate were filled up to 100 mL with deionized water.

2.4 Commercial kits

Table 2.2: Commercial kits used in this study.

Name	Manufacturer	Art. No.
DNeasy® Plant Mini Kit	Qiagen	69104
Live/Dead® BacLight bacterial viability kit	Invitrogen/Molecular Probes	L7012
Mass standards kit for the 4700 Proteomics Analyzer	Applied Biosystems	4333604
Quant-iT™ PicoGreen® dsDNA reagent kit	Invitrogen/Molecular Probes	P7589

2.5 Enzymes

Table 2.3: Enzymes used in this study.

Name	Origin	Specification	Manufacturer	Art. No.
Benzonase	*Serratia marcescens*	Purity > 99 %, 25 U μL^{-1}	Novagen	70664
Glucose-6-phosphate dehydrogenase	*Leuconostoc mesenteroides*	recombinant, expressed in *E. coli*, 550-1,100 U mg^{-1} protein	Sigma	G8529
Proteinase K	*Tritirachium album*	≥ 30 units mg^{-1} protein	Sigma	P6556
Trypsin	Porcine pancreas	Sequencing grade, > 5,000 U mg^{-1} protein	Promega	V5111

2.6 Equipment

Table 2.4: Equipment used in this study.

Instrument	Specification	Manufacturer
2D electrophoresis chamber	Protean II xi cell	Bio-Rad
Analytical scale	BP 210	Sartorius
Analytical scale	BP 1200	Sartorius
Bottle top filters	Non-fibre releasing membran, surfactant-free cellulose acetate, pore size 0.2 µm	Nalgene
Calibrated Imaging Densitometer	GS710	Bio-Rad
Centrifuge	5415D	Eppendorf
Confocal laser scanning microscope	Axiovert 100M	Zeiss
Cooling centrifuge	Biofuge Fresco	Heraeus instruments

Table 2.4: *Continued*

Instrument	Specification	Manufacturer
DGGE system	DCode	Bio-Rad
Dialysis tubing	SpectraPor 3, MWCO: 3500 Da	Serva
Dialysis tubing	Visking, MWCO: 12 – 14 kDa	Serva
Disposable cuvettes	PMMA, 4 clear faces	VWR
Electrophoresis chamber	HE33 horizontal Subarine unit	Amersham Bioscience
Epoxy-coated 8-well diagnostic slides		Thermo Scientific
Ethylene-propylene-diene monomer (EPDM) coupons (65 Shore)	Comply with physical and chemical requirements of the German KTW guideline for drinking water, but not with the DVGW Code of Practice W270.	Schmitztechnik GmbH
External laser	For Molecular imager FX pro plus	Bio-Rad
Fluorescence microscope	Leitz Laborlux	Leitz Wetzlar Germany
Fluorometer	SFM25 Bio-TEK	Kontron Instruments
Freeze dryer	Alpha 1-2	Christ
GelBond PAG film for polyacrylamide gels		GE Healthcare
Gradient delivery system	Model 475	Bio-Rad
Hybridisation oven		Thermo electron cooperation
IEF cell	Protean IEF cell	Bio-Rad
Incubator (20 °C)	ICE 400-800	Memmert GmbH+Co. KG
Incubator (30 °C)	Kelvitron t	Heraeus
Incubator (37 °C)	Kelvitron t	Heraeus
Laser scanner	Molecular imager FX pro plus	Bio-Rad
MALDI TOF/TOF Analyzer	4800 Plus	Applied Biosystems, MDS Sciex
Manifold vacuum stainless steel filtration module		Millipore
Mastercycler	Ep gradient S	Eppendorf

Table 2.4: *Continued*

Instrument	Specification	Manufacturer
Mini BeadBeater		Biospec Products
Molecular Imager Gel Doc	Universal Hood II	Bio-Rad
PCR cycler	Mastercycler epgradient S	Eppendorf
pH meter	WTW (ph 549 ELP)	MultiCal
Phase contrast microscope	Leica DM LS	Leica Microsystems
Plate Reader	Infinite Pro M200	Tecan
Polycarbonate membrane filters	Pore size 0.2, Black	Millipore
Power Pack	P25	Biometra
Reaction chambers for fluorescence *in situ* hybridization		Vermicon
Rocking Platform	WT15	Biometra
Shaking water bath	GFL 1092	Gesellschaft für Labor-Technik mbH
Sorvall centrifuge	Sorvall RC26 Plus	Sorvall Products
Spectrophotometer	Cary 50 Bio	Varian
Syringe filters	Filtropur S plus 0.2, pore size 0.2 µm	Sarstedt
Thermo Scientific diagnostic slides	Epoxy-coated 8-well 6 mm	Menzel
Thermomixer comfort		Eppendorf
Thoma counting chamber		Optik Labor
Tygon tubing	Inner Ø 6.4 mm, outer Ø 9.6 mm, wall 1.6 mm	Saint-Gobain Performance Plastics
Vacuum centrifuge	RVC 2-25	Christ
ZipTip C18	Tip size P10	ZTC185096
Zymogram blue casein gels	Novex, 4 - 16 %, 1.0 mm	EC6415BOX
Zymogram gelatin gels	Novex, 12 %, 1.0 mm	EC6175BOX

2.7 Software and databases

Table 2.5: Software and databases used in this study.

Program	Version or link	Manufacturer
AB SCIEX Data Explorer software	http://www.matrixscience.com/	Matrix Science
AxioVision	3.1	Zeiss
Cary Win UV Simple Reads Application	02.00(25)	Varian
CELLO v.2.5: subCELlular LOcalization predictor	http://cello.life.nctu.edu.tw/	Molecular Bioinformatics Center, National Chiao Tung University (Yu *et al.*, 2006)
Compute pI/Mw tool	http://web.expasy.org/compute_pi/	ExPASy Bioinformatics Resource Portal
i-control	1.8.50.0	Tecan
LSM Image Browser	4.0.0.157	Zeiss
Mascot – peptide mass fingerprint software	http://www.matrixscience.com/	Matrix Science (Perkins *et al.*, 1999)
NCBI protein database	http://www.ncbi.nlm.nih.gov/protein	NCBI
PDQuest	8.0.1	Bio-Rad
Quantity One	4.6.3	Bio-Rad
UniProtKB protein knowledgebase	http://www.uniprot.org/	UniProt

3. METHODS

3.1 Cultivation of drinking-water biofilms

Drinking water biofilms were grown on coupons (72 mm x 26 mm x 2 mm) made of the elastomeric material ethylene-propylene-diene monomer (EPDM 65 Shore; Schmitztechnik GmbH, Germany) at five different locations. The locations were plumbing system A and distribution system A, both supplied with the same drinking water originating from water supplier A, plumbing system B and distribution system B, which were supplied with the same mixture of water originating from water supplier B, or plumbing system C which was supplied by water supplier C (Tab. 3.1). A sampling site prior to entering plumbing system C could not be realized.

Table 3.1: Locations and drinking water installations used for the cultivation of drinking-water biofilms.

Water supplier	Installation	Location
Supplier A	Plumbing system A copper installation, cold water tap	University building Duisburg University of Duisburg-Essen, Duisburg/Germany
	Distribution system A water storage tank, sampling tap	Elevated water tank Duisburg, Drinking water distribution system, Duisburg/Germany
Supplier B	Plumbing system B copper installation, cold water tap	IWW laboratory building, IWW Water Centre, Mülheim an der Ruhr/Germany
	Distribution system B cold water tap at the water meter of the plumbing system	IWW water meter IWW Water Centre, Mülheim an der Ruhr/Germany
Supplier C	Plumbing system C copper installation, cold water tap	University building Essen, University of Duisburg-Essen, Essen/Germany

The EPDM material used as substratum for biofilm growth complied with physical and chemical requirements of the German KTW guideline (Umweltbundesamt, 2011) for organic materials in contact with drinking water, but not with the DVGW Code of Practice W270 (DVGW; Deutscher Verein des Gas- und Wasserfaches, 2007). This material is known to promote biofilm formation (Kilb *et al.*, 2003; Bressler *et al.*, 2009) and was chosen in order to obtain sufficient biomass for EPS isolation and analysis. Before use the coupons were washed in deionized water and pasteurized for 10 min at 80 °C in deionized water. The coupons were vertically inserted into stainless steel reactor vessels (width: 53 mm; height: 95 mm; depth: 37 mm) of 100 mL volume, each providing space for up to 8 coupons (Fig. 3.1). The reactors were connected to cold-water drinking water taps via Tygon® tubing and perfused continuously with drinking water at a flux of 50 mL min^{-1} over cultivation periods of up to 28 d at ambient temperature. Water temperature within the reactors and concentrations of free chlorine were monitored regularly. Free chlorine concentration was determined by the N,N-diethyl-p-phenylenediamine colorimetric method, using a portable spectrophotometer. Drinking water samples were collected for a multi-element analysis from each location in accordance to the standard ISO 19458 (2006). The drinking water samples were taken after 30 min of flushing or, if present, from a continuously running sampling tap, using sterile 50 mL polypropylene centrifuge tubes.

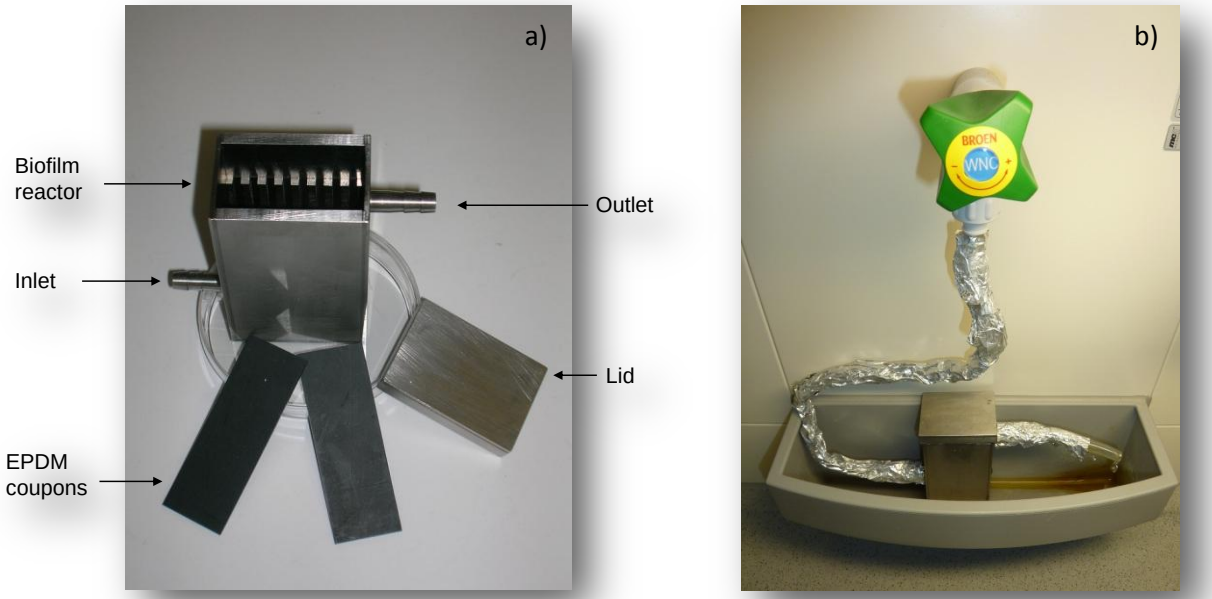

Figure 3.1: a) biofilm reactor with EPDM coupons and b) experimental set up used for the cultivation of drinking-water biofilms.

3.2 Sampling of drinking-water biofilms

Coupons were aseptically removed from the reactors using sterile forceps and drinking-water biofilms were scraped off both sides of the coupons into sterile Petri dishes using a sterile rubber scraper. The biofilms were either directly used for biomass determination or dispersed in 10 mL 6 mM phosphate buffer (pH 7.0) per coupon. The biofilm suspensions were stirred for 5 min for cell dispersal and used for EPS isolation and for microbiological, biochemical or molecular biological analyses.

3.3 Determination of biofilm mass

Wet weight, dry mass and water content of the biofilms were determined according to the standard DIN EN 12880 (2001). A crucible was heated in a muffle furnace at 550 °C for at least 30 min. The crucible was allowed to cool to room temperature (approximately 15 min) in an exsiccator and weighed. 14 d-old drinking-water biofilms from at least 5 EPDM-coupons were scraped off into the crucible and weighed to determine wet weight. To determine the dry weight the crucible containing the biofilm was heated at 105 °C overnight, allowed to cool to room temperature (approximately 5 min) in an exsiccator with P_2O_5 applying vacuum and weighed. The sample was heated again for 1 hour and weighed. The last step was repeated until the sample showed constant mass readings (< 0.5 % (m/m) difference from the previous determination).

The loss and residue on combustion of the dry mass of drinking-water biofilms was determined according to the standard DIN EN 12879 (2001). The crucible containing the dried biofilm mass was combusted in a muffle furnace at 550 °C for 1.5 hours. The crucible was allowed to cool to room temperature (approximately 15 min) in an exsiccator with P_2O_5 applying vacuum and weighed. The sample was reheated to 550 °C for 30 min and weighed. The last step was repeated until the sample showed constant mass readings (< 0.5 % (m/m) difference from the previous determination).

3.4 Multi-element analysis of drinking water and biofilms

Multi-element analyses of all water samples, drinking-water biofilm suspensions or EPS solutions were performed by the IWW Water Centre (Mülheim an der Ruhr, Germany) according to ISO 11885 (2007), using inductively coupled plasma optical emission spectrometry (ICP-OES). Biofilm samples required initial disintegration by acid digestion using a mixture of HNO_3 and H_2O_2 combined with microwave treatment prior to ICP-OES measurement.

3.5 Scanning electron microscopy of drinking-water biofilms

Images of 14 d-old drinking-water biofilms grown on EPDM were acquired by scanning electron microscopy. Coupons with grown biofilms were removed from the biofilm reactor and dried overnight in an exsiccator with P_2O_5 applying vacuum. The coupons were cut into squares of approximately 1 cm^2 and fixed onto specimen stubs. The biofilms were sputtered with gold using a sputter coater (EMITECH K550; Emitech ltd., England). Images were acquired with an environmental scanning electron microscope (ESEM Quanta 400 FEG; FEI Company, USA) coupled to an energy dispersive x-ray micro analysis unit (Genesis 4000 EDS-system; EDAX inc., USA).

3.6 Confocal laser scanning microscopy (CLSM)

Images of fully-hydrated 14 d-old drinking-water biofilms cultivated on EPDM were acquired by CLSM after staining with the Live/Dead® BacLight bacterial viability kit (Invitrogen/Molecular Probes). The fluorescent stains Syto 9 and propidium iodide were mixed and diluted in deionized water to concentrations of 5 µM and 30 µM, respectively. An EPDM coupon with attached 14 d-old drinking-water biofilm was removed from the biofilm reactor and fixed onto a glass slide. 300 µL of the stain mix were applied on the biofilm and the biofilm was incubated for 30 min in the dark in a moist environment. Biofilms were examined at 100 fold (without cover slide) and 1000 fold (with cove slide and immersion oil) magnification. Excitation of the stains was performed with an argon laser and an excitation

wavelength of 488 nm. The applied filters were a band-pass filter for detection of emission in the range of 505 – 530 nm (Syto 9) and a long-pass filter for detection of emission ≥ 560 nm (propidium iodide).

3.7 Microbiological analysis

3.7.1 Total cell count

Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; dihydrochloride, 25 µg ml^{-1} in 2 % formaldehyde). 1 mL of DAPI solution was added to 4 mL of biofilm or cell suspension, or decimal dilutions of the suspensions in sterile particle-free deionized water and incubated for 20 min in the dark at room temperature. Cells were filtered onto black polycarbonate membrane filters (30 mm diameter, 0.2 µm pore size; Millipore) which were stored in the dark at 4 °C until enumeration. Enumeration of cells was carried out with an epifluorescence microscope (Laborlux S; Leitz Germany) at 1000 fold magnification (Objective: PL Fluotar; 100x/1.32 oil; PHACO 3) with the help of a counting grid (100 µm x 100 µm). 20 randomly chosen fields of view of biofilm suspensions showing 20 to 150 cells were examined. Results were given in cells mL^{-1}.

3.7.2 Heterotrophic plate count

Culturable heterotrophic plate count bacteria were determined by the spread plate method on R2A medium (Reasoner and Geldreich 1985). 0.1 mL of biofilm suspension or decimal dilutions of the biofilm suspension in sterile particle-free deionized water were plated in triplicates on R2A medium and the agar plates were incubated for 7 d at 20 °C. Plates showing 30 to 300 colonies were used for enumeration. Results were given in colony-forming units (cfu) mL^{-1}.

3.7.3 Culturability of *P. aeruginosa* pure cultures

Culturability of *P. aeruginosa* AdS pure cultures were determined by spread plate method on nutrient agar. 0.5 mL of cell suspension or 0.1 mL of decimal dilutions of the suspensions in sterile particle free deionized water were plated in triplicates on nutrient agar plates and the

agar plates were incubated for 48 h at 37 °C. Plates showing 30 to 300 colonies were used for enumeration. Results were given in cfu mL^{-1}.

3.7.4 Culturability of *P. aeruginosa* in biofilms

Culturability of *P. aeruginosa* AdS present in or incorporated into established drinking-water biofilms was determined by spread plate method on the selective CN agar. 0.5 mL of biofilm suspension or 0.1 mL of decimal dilutions of the suspensions in sterile particle free deionized water were plated in triplicates on CN agar plates and the agar plates were incubated for 48 h at 37 °C. Plates showing 30 to 300 colonies were used for enumeration. Results were given in cfu mL^{-1}.

3.8 Isolation of extracellular polymeric substances (EPS)

Isolation of EPS from drinking-water biofilms was carried out in a miniaturized form compared to isolation procedures commonly applied for activated or sewage sludges (Jahn and Nielsen, 1995; Frølund *et al.*, 1996), using 8 to 20 mL-volumes of biofilm suspensions. The isolation procedures tested were shaking, shaking in presence of a cation exchange resin (CER), NaOH treatment in combination with formaldehyde, EDTA treatment or heating, which were carried out according to Fig. 3.3.

- *Shaking treatment* -

8 mL-volumes of biofilm suspensions were shaken on a Vortex (Genie 2) shaker in 50 mL centrifuge tubes (conical bottom, polypropylene; Sarstedt) for up to 60 min at maximum speed (Fig. 3.2). The Vortex was equipped with a platform head able to hold 4 centrifuge tubes.

- CER isolation -

The CER Dowex (Marathon C, Na^+-form, Sigma) was washed twice in 6 mM phosphate buffer (pH 7.0) by stirring for 2 x 15 min (20 g CER in 100 mL buffer). 1.6 g of hydrated CER were added to 8 mL biofilm suspension in 50 mL centrifuge tubes (conical bottom, polypropylene; Sarstedt) and shaken on a Vortex (Genie 2) shaker for up to 60 min at maximum speed (Fig. 3.2). The Vortex was equipped with a platform head able to hold 4 centrifuge tubes. After treatment the CER was allowed to sediment and the treated biofilm suspension was decanted into a new centrifuge tube.

Figure 3.2: Experimental setup used for EPS isolation by shaking in absence or presence of CER.

- EDTA -

2 % (w/v) EDTA (tetrasodium salt) solution was prepared in deionized water and autoclaved for 20 min at 121 °C. 15 mL Na_4EDTA solution were added to 15 mL biofilm suspension and stirred on a magnetic stirrer for 3 h at 4 °C. Blank samples containing deionized water only were treated accordingly to determine the impact of EDTA on subsequent analyses.

- Formaldehyde/NaOH -

0.12 mL 37 % formaldehyde were added to 20 mL biofilm suspension and stirred on a magnetic stirrer for 1 h at 4 °C. 8 mL of 1 M NaOH were added on top and the suspension was stirred for additional 3 h at 4 °C. Blank samples containing deionized water only were treated accordingly with formaldehyde and NaOH or separately with formaldehyde or NaOH to determine the impact of the chemicals on subsequent analyses.

- Heat -

20 mL biofilm suspension were heated at 70 °C for 1 h in a water bath and briefly stirred on a magnetic stirrer every 15 min.

Subsequently to each isolation treatment the suspensions were centrifuged at 20,000 x g for 20 min at 4 °C. The supernatants were filter sterilized (cellulose acetate filters, pore size 0.2 µm, Sarstedt) and corresponded to the cell free EPS-solutions. The EPS solutions were further dialysed against 3 x 5 L changes of deionized water (2 changes 1 h and 1 change overnight; Spectra/POR 3 dialysis tubing, MWCO 3500 Da, Serva).

For culturability studies cell pellets were resuspended in 6 mM phosphate buffer (pH 7.0) in the initial volume prior to centrifugation. For molecular biological examination cell pellets were resuspended in 2 mL water for molecular biology, transferred into 2 mL Eppendorf tubes and centrifuged at 16,110 x g for 30 min at 4 °C. The supernatant was discarded and the cell pellet was stored at -20 °C.

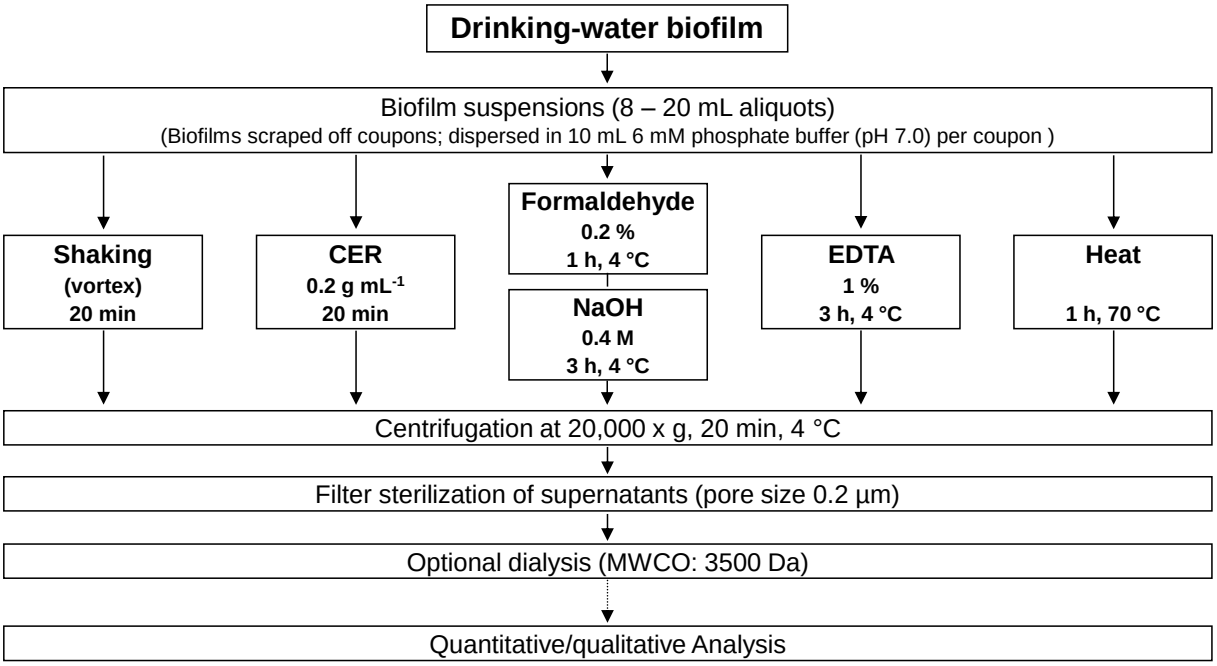

Figure 3.3: Schematic representation of EPS isolation procedures applied for drinking-water biofilms. CER, cation exchange resin; MWCO, molecular weight cut-off.

3.9 Biochemical analysis of biofilms and EPS

3.9.1 Proteins

Proteins were determined with the Lowry assay modified by Peterson (1977) using the commercially available Lowry reagent (Sigma L3540), Folin-Ciocalteu's phenol reagent (Sigma F9252) and bovine serum albumin (Sigma P5619). The following reagents were prepared according to the manufacturer's instructions:

- Lowry reagent:

  The content of a vial containing 2 g Lowry reagent (composition according to the manufacturer: 45.03 % sodium dodecyl sulfate, 38.49 % sodium carbonate, 15.13 % lithiumhydroxide-monohydrate, 1.35 % copper-tartrate-complex) was dissolved in 40 mL deionized water by stirring.
- Folin-Ciocalteu's phenol reagent:

  18 mL Folin-Ciocalteu's phenol reagent (composition according to the manufacturer: 61.2 % water, 12.2 % lithium sulfate, 2 % sodium tungstate dehydrate, 9.5 % hydrochloric acid (≥ 25 %), 6.9 % phosphoric acid solution in water, 2 % sodium molybdate-dihydrate) were mixed with 90 mL deionized water.

0.5 mL sample, deionized water (blank) or standards were mixed with 0.5 mL Lowry reagent, weakly shaken and incubated for 20 min at room temperature. 0.25 mL Folin-Ciocalteu's phenol reagent were added, weakly shaken and the mixture was incubated for 30 min at room temperature. The solutions were transferred into a semi-micro cuvette and absorbance was measured against deionized water at 750 nm. If required, samples were diluted in deionized water in test tubes. A 3-point calibration was carried out with BSA standards covering a concentration range between 0 µg mL^{-1} (blank) and 60 µg mL^{-1} of protein. The determination was performed in triplicates.

3.9.2 Carbohydrates

Carbohydrates were determined with the sulfuric acid/phenol method according to Dubois *et al.* (1956). The following solutions were prepared:

- 5 % phenol solution:

  25 g phenol were dissolved in 500 mL deionized water.

2.5 mL sulfuric acid (95 % - 97 %) and 0.5 mL phenol solution were added to 0.5 mL sample, deionized water (blank) or standards. The samples were incubated for 10 min at room temperature, further incubated in a water bath for 15 min at 30 °C and allowed to cool down to room temperature for 5 min. The solutions were transferred into a macro cuvette and absorbance was measured against deionized water either at 490 nm in case of neutral polysaccharides or 480 nm in case of acidic polysaccharides. If required, samples were diluted in deionized water in test tubes. A 3-point calibration was carried out either with D(+)glucose standards for neutral polysaccharides or D-glucuronic acid standards for acidic polysaccharides covering a range between 0 µg mL^{-1} (blank) and 75 µg mL^{-1} carbohydrate, or 0 µg mL^{-1} (blank) and 150 µg mL^{-1} carbohydrate, respectively. The determination was performed in triplicates.

3.9.3 Uronic acids

Determination of uronic acids was performed according to Filisetti-Cozzi and Carpita (1991). The following solutions were prepared:

- 4 M sulfamate solution:

  19.42 g sulfamic acid were added to 20 ml deionized water. A saturated potassium KOH solution was added drop wise to the solution for complete dissolution of the salts (approximately 8 mL). The solution was allowed to cool to room temperature. The pH value was adjusted to pH 1.6 with saturated KOH solution and the solution was filled up to 50 mL with deionized water.
- Sulfuric acid/tetraborate solution:

  5.72 g di-sodium tetraborate-decahydrate was dissolved in 200 mL sulfuric acid (95 % - 97 %).
- 0.15 % m-hydroxybiphenyl solution:

  15 mg m-hydroxybiphenyl were dissolved in 10 mL 0.5 % NaOH.

40 µL of sulfamate solution were added to 0.4 mL of samples, deionized water (blank) or standards, respectively, and mixed thoroughly. 2.4 mL of sulfuric acid/tetraborate solution were added. The solutions were shaken and then incubated in a water bath for 20 min at 98 °C. The solutions were cooled for 5 min on ice. 80 µL m-hydroxybiphenyl solution were added, the mixtures were thoroughly stirred, and incubated for 10 min at room temperature. Absorbance was measured with a spectrophotometer at 525 nm. If required, samples were diluted in deionized water in test tubes. A 3-point calibration was prepared with D-glucuronic acid standards covering a range between 0 µg mL^{-1} (blank) and 150 µg mL^{-1} uronic acid. The determination was performed in triplicates.

3.9.4 DNA

DNA was determined with the commercially available Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Invitrogen/Molecular Probes). The following reagents were prepared according to the manufacturer's instructions:

- 1 x TE buffer:

  20 x TE buffer concentrate (composition: 200 mM Tris-HCl, 20 mM EDTA, pH 7.5; Invitrogen/Molecular Probes) was diluted 20 fold in water for molecular biology (DEPC-treated).
- PicoGreen reagent:

  The PicoGreen reagent concentrate was diluted 200 fold in 1 x TE buffer.

1 mL of PicoGreen reagent was added to 1 mL of sample, 1 x TE buffer or standard in single use macro cuvettes (4 clear sides) and the mixtures were incubated for 2 min at room temperature. Relative fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. If required, samples were diluted in TE buffer in cuvettes. A 4-point calibration was prepared with λ-DNA (Invitrogen/Molecular Probes) standards in TE buffer (1 x) covering a high range between 0 µg mL^{-1} (blank) and 2 µg mL^{-1} DNA, or low range between 0 µg mL^{-1} (blank) and 50 ng mL^{-1} for samples with low DNA concentrations.

3.9.5 2-keto-3-deoxyoctonate (KDO)

KDO was determined according to Karkhanis *et al.* (1977). The following reagents were prepared:

- 0.1 M H_2SO_4:
  0.543 mL concentrated H_2SO_4 (98 %) were filled up to 100 mL with deionized water.
- 0.04 M HIO_4 in 0.0625 M H_2SO_4:
  0.912 g HIO_4 were added to 0.34 mL concentrated H_2SO_4 (98 %) and filled up to 100 mL with deionized water.
- 2.6 % (w/v) $NaAsO_2$ in 0.5 M HCl:
  4.2 mL concentrated HCl (36 %) were added to 2.6 g $NaAsO_2$ and filled up to 100 mL with deionized water.
- 0.6 % thiobarbituric acid (TBA):
  0.6 g TBA were dissolved in 100 mL deionized water.

0.25 mL sample, deionized water (blank) or standards were mixed with 0.25 mL 0.1 M H_2SO_4 and heated at 100 °C for 30 min. The suspensions were allowed to cool to room temperature and centrifuged at 8,000 x g for 5 min. 0.25 mL were transferred into a new 2 mL Eppendorf tube, mixed with 0.125 mL 0.04 M HIO_4 in 0.0625 M H_2SO_4 and incubated for 20 min at room temperature. 0.125 mL 2.6 % $NaAsO_2$ in 0.5 M HCl were added and mixed until the brown color disappeared. The sample suspensions were mixed with 0.25 mL 0.6 % TBA, heated at 100 °C for 15 min and, while still hot, mixed with 0.5 mL DMSO. The samples were allowed to cool to room temperature before absorbance was measured with a spectrophotometer at 548 nm. A 3-point calibration was prepared with KDO standards covering a range between 0 µg mL^{-1} (blank) and 30 µg mL^{-1} KDO. The determination was performed in duplicates.

3.10 Enzyme activity measurements

3.10.1 Glucose-6-phosphate dehydrogenase activity

Suspensions of 14 d-old drinking-water biofilms, drinking-water biofilm cell extracts and EPS isolated by shaking or CER treatment were analyzed for presence of activity of the strictly

intracellular enzyme G6PDH. The measurement was performed according to Ng and Dawes (1973) in a miniaturized form using 96-well microtitre plates. Cell extracts were obtained by ultrasound treatment (6 x 10 s intervals with 1 min pauses between the intervals; 40 W; on ice keeping the temperature below 40 °C) of cells obtained after EPS isolation and were applied to confirm presence of G6PDH within biofilm cells. 0.1 µg mL^{-1} and 1 µg mL^{-1} G6PDH standards from *Leuconostoc mesenteroides* (Sigma) were used as positive control. Negative controls were prepared by incubating the biofilm suspensions, the cell extract suspensions and EPS solutions for 30 min at 98 °C.

Optional 20 fold concentration of all samples and controls was carried out by pipetting 200 µL of each sample or control into designated wells of the microtitre plate, vacuum-centrifugation until dry and resuspension in 10 µL sterile deionized water.

The following solutions were prepared:

- 120 mM Tris/HCl-buffer (pH 8.6):
  1.45 g Tris were dissolved in deionized water and pH was adjusted with 1 M HCl.
- 250 mM $MgCl_2$ solution:
  5.08 g $MgCl_2$ x H_2O were dissolved in 100 mL deionized water.
- 20 mM glucose-6-phosphate solution:
  0.045 g glucose-6-phosphate were prepared fresh in 8 mL deionized water.
- 10 mM β-NADP solution:
  0.045 g of β-NADP were prepared fresh in 6 mL deionized water to obtain a 10 mM β-NADP solution.
- Substrate solution:
  0.5 mL Tris/HCl-buffer (120 mM; pH 8.6), 0.375 mL glucose-6-phosphate solution (20 mM), 0.25 mL β-NADP solution (10 mM), 0.09 mL $MgCl_2$ solution (250 mM) and 0.235 mL deionized water were combined per analyzed sample.

10 µL sample, heat inactivated sample (negative control), G6PDH standard (positive control) and deionized water (blank) were transferred into designated wells of a 96-well microtitre plate. 0.29 µL substrate solution preheated to 37 °C were added to each well and the microtitre plate was placed into a multimode microtitre plate reader (Infinite Pro 200, Tecan). Absorbance was measured immediately after addition of the substrate solution at

1 min intervals over a period of 3 h at 340 nm. The plate reader was set to 5 s orbital shaking with 1.5 mm amplitude (335.8 rpm) prior to each measurement cycle. All samples and controls were measured in triplicates. Appropriate arrangement of the microtitre plates allowed for simultaneous measurement of all samples from two independent reactor runs.

3.10.2 Protease activity determination by zymogram gels

Protease activity was qualitatively determined by 1D gel electrophoresis using Novex 4 % - 16 % Zymogram Blue-Casein gels containing 0.1 % casein as substrate, or Novex 10 % Zymogram Gelatin gels containing 0.05 % gelatin as substrate. 15 µL of biofilm suspension, cell fraction, or EPS fraction were mixed with 5 µL non-reducing Roti®-Load 2 (4 x) sample buffer (Roth), incubated for 5 min at room temperature and transferred into a well of the gel. 20 µL Seeblue pre-stained marker were applied into one well for molecular weight comparison. The gel electrophoresis was carried out for 90 min at 125 V in 1 x Rotiphorese SDS running buffer (Roth). After electrophoresis gels were removed from the cassette and incubated for 2 x 1 h in 1x Zymogram renaturing buffer at room temperature with gentle agitation. The gels were transferred into 1 x Zymogram developing buffer and incubated for 30 min at room temperature with gentle agitation. Gels were transferred into fresh developing buffer and incubated overnight at 37 °C. Zymogram gelatin gels were stained with the Simply Blue Safe Stain (Invitrogen/Molecular Probes) according to the manufacturer's instructions (Section 3.13.4). Blue-casein gels did not require staining.

3.10.3 Fluorometric determination of enzyme activity

Drinking-water biofilms, isolated EPS and biofilm cells after EPS isolation were subjected to enzyme activity measurements using commercially available 4-methoxy-ß-naphthylamide or methylumbelliferyl (MUF) substrates (Sigma-Aldrich). A microtitre plate assay was designed to be able to analyze a variety of enzymes at once (Fig. 3.4). The substrates were L-leucine-4-methoxy-β-naphthylamide (wells: A1 – A12), 4-MUF-α-D-glucopyranoside (wells: B1 – B12), 4-MUF-β-D-glucopyranoside (wells: C1 – C12), 4-MUF-N-acetyl-β-D-glucosaminide (wells:

D1 – D12), 4-MUF-stearate (wells: E1 – E12), 4-MUF-butyrate (wells: F1 – F12) and 4-MUF-phosphate (wells: G1 – G12).

2 mM substrate stock solutions were prepared in 2-methoxyethanol. 10 µL of each substrate were transferred into designated wells of a microtitre plate (Fig. 3.4). 190 µL of biofilm suspension (wells: A1 – G3), biofilm cells (wells: A4 – G6), or EPS (wells: A7 – G9) were added into designated wells and mixed thoroughly by aspiration. Heat inactivated controls were run using biofilms (wells: A10 – G10), biofilm cells (wells: A11 – G11), or EPS (wells: A12 – G12), which were previously treated at 98 °C for 30 min. No substrate controls were measured using 10 µL of 2-methoxyethanol without substrate and 190 µL of biofilm suspension (well: H8), biofilm cells (well: H9), or EPS (well: H10).

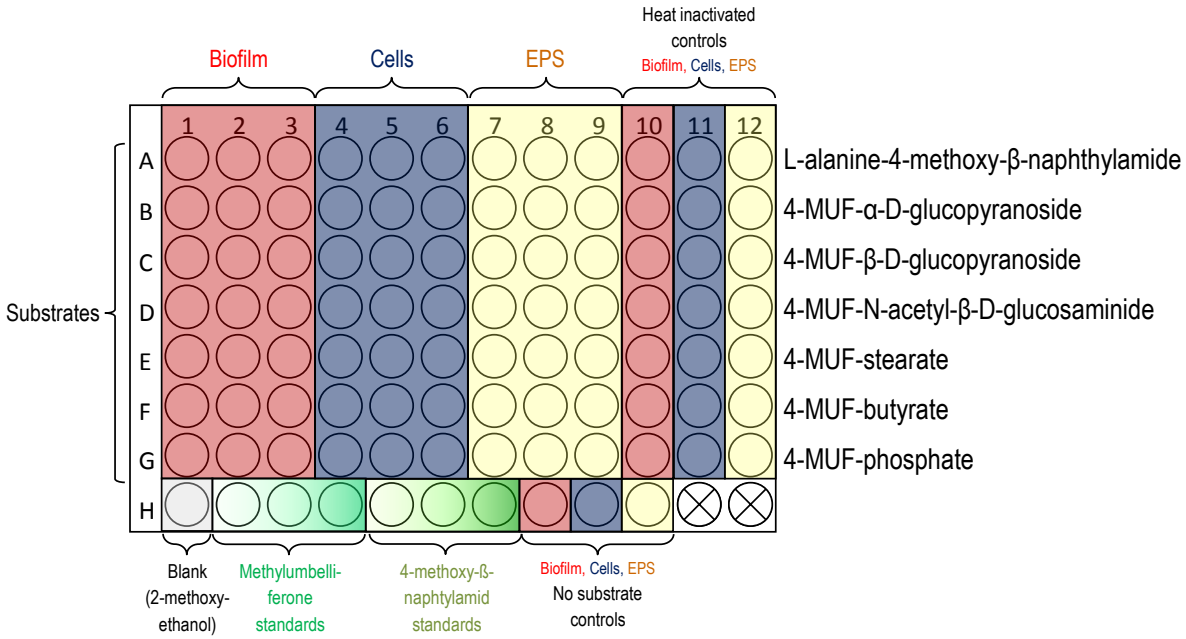

Figure 3.4: Pipetting scheme used for the fluorometric determination of enzyme activity in a microtitre plate format. MUF, methylumbelliferyl.

3-point calibrations were performed using methylumbelliferone (wells: H2 – H4) or 4-methoxy-ß-naphthylamide (wells: H5 – H7) covering a range of 0 mM (blank; well H1) to 200 mM or 0 mM (blank; well H1) to 100 mM, respectively. The arrangement of the microtitre plate allowed for a simultaneous triplicate determination of enzyme activities of seven enzyme classes in a biofilm sample, biofilm cells and isolated EPS. Fluorescence was measured immediately after addition of samples with a multimode microtitre plate reader

(Infinite Pro 200, Tecan). The plate reader was set to successive measurements at an excitation wavelength of 360 nm and emission readings at 450 nm, followed by excitation at 330 nm and emission readings at 420 nm. Measurements were performed at intervals of 1 min for 2 h at ambient temperature (approximately 26 °C). The plate reader was set to 5 s orbital shaking with 1.5 mm amplitude (335.8 rpm) prior to each measurement cycle.

3.11 Molecular biology methods

3.11.1 Sample preparation for population analysis by DGGE

DGGE was applied for the analysis of population diversity in drinking-water biofilms. 16 mL of biofilm suspension were centrifuged at 20,000 x g for 20 min at 4 °C. Supernatants were discarded, cell pellets were resuspended in 2 mL water for molecular biology, transferred into 2 mL centrifuge tubes and centrifuged at 16,110 x g for 30 min. The supernatants were discarded and cell pellets were stored at -20 °C.

3.11.2 DNA Isolation

DNA from cell or EPS pellets was isolated by use of the DNeasy Plant Mini Kit (Qiagen) with a modified procedure of the manufacturer's protocol for purification of total DNA from plant tissue ("Mini Protocol"). Pellets were resuspended in 400 µL buffer AP1 and transferred into Rotilabo®-microcentrifuge tubes with screw cap containing 250 µg of sterile glass beads (diameter ≤ 106 µm; Sigma). The samples were shaken in a Mini BeadBeater for 3 min at 4,800 rpm and transferred into 2 mL centrifuge tubes. 4 µL RNase A stock solution (100 mg mL^{-1}) were added and the mixture was incubation in a thermomixer (Eppendorf) for 10 min at 65 °C with 15 s shaking at 1,000 rpm every 2.5 min. 130 µL buffer AP2 were added and the samples were incubated for 5 min on ice. The cell lysate was centrifuged for 4 min at 16,110 x g. The supernatant was transferred onto QIAshredder Mini spin columns placed in 2 mL collection tubes and centrifuged for 4 min at 16,110 x g. The filtrate was transferred into a new tube. 1.5 volumes of buffer AP3/E were added and mixed immediately. 650 µL of the mixture were pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube

and centrifuged for 1 min at 6,000 x g to load DNA onto the column. The eluate was discarded and the previous step was repeated with the remaining sample. 500 µL buffer AW were added and centrifuged for 1 min at 6,000 x g. The eluate was discarded and another 500 µL buffer AW were added and centrifuged for 4 min at 16,110 x g. DNA was eluted from the column into 1.5 mL tubes in two steps, each time incubating the column in 100 µL of buffer AE (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) for 5 min and centrifugation for 1 min at 6,000 x g. Isolated DNA was stored at -20 °C until use.

3.11.3 Amplification of 16S rDNA fragments

Bacterial 16S rDNA fragments of isolated DNA were amplified according to Bressler *et al.* (2009) by touchdown polymerase chain reaction (PCR) using the primer pair 27f_GC and 517r (Tab. 3.3). A PCR master mix was prepared according to Tab. 3.2. 1 µL of DNA sample was mixed with 49 µL of PCR master mix in 0.2 mL reaction tubes. PCR consisted of an initial denaturation step at 94 °C (1 min), 10 cycles of 94 °C (1 min), 71 °C (1 min) decreasing by 1 °C per cycle to 61 °C, and 72 °C (1.5 min), followed by 19 cycles of 94 °C (1 min), 61 °C (1 min) and 72 °C (1.5 min), and one final elongation cycle of 72 °C (7 min). DNA concentration was quantified with Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen/Molecular Probes) (Section 3.9.4).

Table 3.2: PCR master mix composition for one reaction.

Component	Concentration	Final Concentration	Volume per sample [µL]
Taq-Master	5 x	1 x	10
PCR buffer	10 x	1 x	5
dNTPs Mix	10 mM	200 µM	1
27f_GC primer	10 µM	0.5 µM	2.5
517r primer	10 µM	0.5 µM	2.5
Taq-Polymerase	5 U μL^{-1}	2.5 U	0.5
H_2O (for mol. biology)	-	-	27.5
Final Volume	-	-	49

Table 3.3: Primers applied for 16S rDNA amplification.

Primer	Sequence	Reference
27f_GC	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CAG AGT TTG ATC (A/C)TG GCT CAG-3'	Murray *et al.* (1996) (GC-clamp) Kilb *et al.* (1998) (underlined)
517r	5'-ATT ACC GCG GCT GCT GG-3'	Murray *et al.* (1996)

3.11.4 Agarose gel electrophoresis

The PCR products were analyzed on a 1 % agarose gel to confirm amplification and correct size of the amplicon. 5 µl of PCR product were mixed with 1 µl TriTrack (6 x) loading dye (Fermentas) and pipetted into wells of the agarose gels. 5 µl of Mass Ruler DNA ladder (Fermentas) were added into one well of each gel. The gel electrophoresis was run for approximately 50 minutes at 100 V in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3; Bio-Rad). Gels were stained with ethidium-bromide (Section 3.13.2) and examined with the Molecular Imager Gel Doc, Universal Hood II (Bio-Rad).

3.11.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using 7.5 % acrylamide gels (16 cm x 14 cm x 0.1 cm) with a 40 % to 60 % denaturation gradient (100 % denaturant corresponds to 7 M urea and 40 % formamide). 14 mL denaturant solutions of 40 % and 60 %, respectively, were prepared according to Tab. 3.4.

Table 3.4: Composition of 40 % and 60 % denaturant solutions for DGGE.

Component	40 % denaturant solution	60 % denaturant solution
0 % denaturant (7.5 % acrylamide)	8.4 mL	5.6 mL
100 % denaturant (7 M urea, 40 % formamide, 7.5 % acrylamide)	5.6 mL	8.4 mL

140 µl of 10 % (w/v) APS were added to the solutions. Polymerization of the gels was started by addition of 9 µL or 18 µL TEMED (N,N,N',N'-tetracethylethylendiamine) to the 40 % or 60 % denaturant solution, respectively. Gels were poured using a gradient delivery system

(Model 475, Bio-Rad), overlaid with aqueous 2-butanol and allowed to polymerize for 3 to 4 hours. After polymerization the 2-butanol phase was removed and a stacking gel (5 mL 7.5 % acrylamide, 60 µL 10 % APS, 6 µL TEMED) was poured on top of the separating gel and allowed to polymerize. Samples were mixed with TriTrack (6 x) loading dye (Fermentas) and volumes containing 500 ng – 750 ng of DNA were loaded into the wells of the gel. Electrophoresis was carried out for 17 hours at 70 V and 58 °C in 1 x TAE buffer (Bio-Rad) in a DCode™ Universal Mutation Detection System (Bio-Rad). The gels were stained either with the silver staining protocol according to Blum *et al.* (1987) (Section 3.13.3) or with Sybr®Gold (Invitrogen/Molecular Probes) (Section 3.13.5).

DGGE band patterns were evaluated by counting the number of bands of each sample. Similarity of band patterns was analyzed by calculating the Dice coefficient C_s according to the following equation:

$$C_s = 2j / (a + b)$$

With: j = number of similar bands in two samples (A and B)
a = number of samples in sample A
b = number of samples in sample B

3.11.6 Fluorescence *in situ* hybridization

Detection of *P. aeruginosa* in drinking-water biofilms by FISH was performed by use of the Psae 16S-182 probe (Wellinghausen *et al.*, 2005). 2 mL of the drinking-water biofilm suspension were transferred into 2 mL Eppendorf tubes and centrifuged for 10 min at 6,000 x g and 4 °C. The supernatant was discarded and the cells were resuspended in 2 mL 4 % paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). The suspension was incubated for 1 h at 4 °C. The cell suspension was centrifuged for 5 min at 6,000 x g and 4 °C. The supernatant was discarded and the cell pellet was washed with 2 mL PBS. The cell suspension was centrifuged for 5 min at 6,000 x g and 4 °C. The supernatant was discarded and the cells were resuspended in a 1:1 (v/v) mixture of PBS and ethanol and stored at -20 °C until further use. 10 µL of the fixed sample were pipetted into a well of an epoxy-coated

8-well diagnostic slide (Thermo Scientific) and air-dried. The slide was successively submerged in 50 %, 80 % and 96 % ethanol solutions for 3 min each for dehydration and air-dried. The fixed and dehydrated cells were hybridized by addition of 10 µL hybridization buffer containing 5 µg mL^{-1} of the Psae 16S-182 probe. Hybridization was carried out in a humid reaction chamber (Vermicon) for 90 min at 46 °C. The slide was transferred into a reaction chamber containing 25 mL washing buffer, preheated to 46 °C, and incubated for 15 min at 46 °C to remove unbound probe. The slides were washed with deionized water and air-dried. Cells were counterstained by addition of 10 µL DAPI (1 µg mL^{-1}) solution to the sample and incubation for 20 min at room temperature in the dark. The slides were washed in deionized water and stored at 4 °C until enumeration. Enumeration of cells was carried out with an epifluorescence microscope (Laborlux S; Leitz Germany) at 1,000 fold magnification (Objective: PL Fluotar; 100x/1.32 oil; PHACO 3) with the help of a counting grid (100 µm x 100 µm). FISH-positive and DAPI stained cells were enumerated in the same 20 randomly chosen fields of view successively applying the Cy3 and DAPI filter, respectively. The ratio of FISH-positive and DAPI stained cells was determined, and concentration of FISH-positive cells was calculated by multiplication of the ratio with total cell counts as determined by the DAPI method as described in Section 3.7.1.

3.12 Analysis of proteins by 2D gel electrophoresis

3.12.1 Sample preparation

EPS proteins of drinking-water biofilms were separated by 2D gel electrophoresis (2DE). EPS samples isolated from drinking-water biofilms as described in Section 3.8 required additional preparatory steps, since preliminary experiments showed that eDNA and high contents of inorganic cations interfered with the isoelectric focusing step of the 2DE (Fig. 4.6). EPS solutions were treated with the DNase Benzonase to remove interference caused by eDNA. 1 mL Benzonase buffer (500 mM Tris, 10 mM $MgCl_2$, pH 8, filter sterilized) were added to 9 mL aliquots of EPS solution. 10 µL of Benzonase (from *Serratia marcescens;* purity > 99 %; Novagen) corresponding to final concentration of 65 U mL^{-1} were added, mixed thoroughly and incubated at 37 °C for 1 h. Preliminary experiments indicated that these conditions

resulted in degradation of DNA by > 99.5 %. Benzonase treated EPS solutions were dialysed against

3 x 5 L changes of deionized water (2 changes 1 h and 1 change overnight; Visking dialysis tubing, MWCO 12 - 14 kDa, Serva). The protein concentration of benzonase-treated and dialysed EPS solutions was determined with the Lowry assay (Section 3.9.1). Aliquots containing the desired protein content were freeze-dried.

3.12.2 Isoelectric focusing (IEF)

Freeze-dried EPS samples were dissolved in 380 µL IEF buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 5 mM tributylphosphine, 0.25 % Servalyt 3 – 10 ampholyte, few crystals bromophenol blue prepared in ultrapure water; Ultrapur, Merck) and incubated for 1 h at room temperature. 330 µL of the sample solutions were pipetted along channels of an IEF tray. IPG strips (immobilized pH gradient; pH 3 – 10 linear, Bio-Rad) were placed gel-side down into the sample-containing channels and each strip was covered with 4 mL mineral oil. The IPG strips were passively rehydrated with the sample solution overnight (approximately 17 hours) at 20 °C. After rehydration electrode-wicks (Bio-Rad) were moistened with ultrapure water (Ultrapur, Merck) and placed below the IPG strips onto the electrodes of the IEF tray. The IEF was run at 20 °C with a maximal amperage of 75 µA per IPG strip using the focusing program as shown in Tab. 3.5.

Table 3.5: Program applied for the IEF of EPS proteins from drinking-water biofilms.

Step	Voltage	Mode	Duration
1	200 V	Rapid increase	45 min
2	500 V	Rapid increase	45 min
3	1,000 V	Rapid increase	45 min
4	10,000 V	Linear increase	4 h
5	10,000 V	Rapid increase	approximately 5 h

The IEF was carried out until approximately 75 kVh were reached. Electrode-wicks were exchanged regularly (at least between each step) to allow desalting the samples and enhance current flow. Each step of the program was monitored and if necessary extended until the amperage was below 45 µA per IPG strip prior to switching to the next step. IPG strips were either directly applied for 2nd dimension separation of proteins or stored at -20 °C for up to 1 week.

3.12.3 Preparation of SDS-Tris-glycine gels

Proteins were separated by size in the 2nd dimension on 12 % polyacrylamide SDS-Tris-glycine gels. Cassettes each composed of a pair of glass plates, 1 mm spacers and screw clamps were thoroughly cleaned with deionized water and acetone and assembled. Per gel (20 cm x 20 cm) 17 mL deionized water, 12.5 mL 1.5 M Tris/HCl pH 8.8, 20 mL 30 % (w/v) acrylamide/bisacrylamide solution (ratio 29:1), 0.5 mL 10 % (w/v) SDS, 25 µL TEMED and 75 µL 40 % (w/v) APS were prepared and poured in the space formed between the plates of the cassette. 3 mL water-saturated 2-butanol were pipetted on top of the gel to form an even surface and to protect the gels from drying. The gels were allowed to polymerize for 3 h at room temperature and stored overnight at 4 °C.

3.12.4 Gel electrophoresis of EPS proteins

IPG strips were equilibrated on a rocking platform (WT 15, Biometra) set to slight tilting for 10 min submerged in 10 mL equilibration buffer (6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris/HCl pH 8.8) containing 0.1 g dithiothreitol in the first step and for another 10 min in equilibration buffer containing 0.5 g iodoacetamide in the second step.

Residual 2-butanol was removed from the gel surface and the surface was washed three times with 1 x SDS-Tris-glycine running buffer (0.192 M glycine, 0.025 M Tris, 0.1 % (w/v) SDS; Roth). Equilibrated IPG strips were briefly washed in the running buffer and applied onto of the upper gel surface. An electrode-wick was wetted with 5 µL protein marker (Mark12, Invitrogen) and placed in a corner on the gel surface. The IPG strip and the electrode-wick were fixed with 0.5 % (w/v) agarose containing a few crystals of

bromophenol blue, avoiding entrapment of air. Electrophoresis was performed at 20 mA per gel for 45 min, followed by 35 mA per gel for 4.5 h in a Protean II Xi cell (Bio-Rad) and 1 x SDS-Tris-glycine running buffer (Roth). Gels were stained with the Coomassie-Brilliant Blue protocol (Kang et al., 2002), SyproRuby protocol, or according to the silver staining protocol by Blum *et al.* (1987) (Section 3.13).

3.12.5 MALDI-TOF-MS

MALDI-TOF-MS was applied to identify EPS proteins which were previously separated by 2DE. The analysis required a clean-up and trypsin digestion of the selected protein spots using the following solutions and steps.

Solutions:

- 30 mM potassium-hexacyanoferrate
  0.198 g $K_3[Fe(CN)_6]$ were dissolved in 20 mL double distilled water
- 100 mM sodium thiosulfate
  0.496 g $Na_2S_2O_3$ were dissolved in 20 mL double distilled water
- 10 mM ammonium hydrogencarbonate buffer
  77 mg NH_4HCO_3 were dissolved in 100 mL double distilled water. The solution was prepared fresh on the day of clean-up.
- 5 mM dithiothreitol in 10 mM ammonium hydrogencarbonate
  15.4 mg dithiothreitol were dissolved in 20 mL 10 mM NH_4HCO_3 buffer. The solution was prepared fresh on the day of clean-up.
- 55 mM iodoacetamide in 10 mM ammonium hydrogencarbonate
  102 mg iodoacetamide were dissolved in 10 mL 10 mM NH_4HCO_3 buffer. The solution was prepared fresh on the day of clean-up
- Acetonitrile
- 25 ng μL^{-1} trypsin working solution
  A 100 ng μl^{-1} trypsin (from porcine pancreas, Promega) stock solution was prepared in 1 mM acetic acid. The stock solution was diluted 4 fold in 10 mM NH_4HCO_3 buffer immediately prior to use.
- Matrix solution

10 mg mL^{-1} a-cyano-4-hydroxycinnamon acid (CHCA) were prepared in acetonitrile and 0.1 % trifluoroacetic acid.

- Calibration standard

  The mass standards kit for the 4700 proteomics analyzer (Applied Biosystems, 4333604) was used for calibration. The calibration mixture was composed of β-galactosidase digested des-Arg1-Bradykinin, Angiotensin I, Glu1-Fibrinopeptide B, ACTH (1-17) and ACTH (18-39) suspended in a CHCA matrix.

Sample clean-up and digestion:

All instruments were thoroughly rinsed with acetone and allowed to dry. 1 mL single use plastic pipette tips were used to excise spots from the 2D gels. Before use the pipette tips were cut to obtain an opening of approximately 2 mm in diameter. Excised gel plugs were transferred into 1.5 mL Eppendorf tubes. 50 µL of a 10 % acetic acid solution were added and the gel plugs were stored at 4 °C until clean-up. The clean-up was carried out in a heating block set to 21 °C and 500 rpm shaking if not otherwise indicated. Solutions were removed between each step.

1. The gel plugs were washed twice in 50 µL of a 1:2 (v/v) $K_3[Fe(CN)_6]$ and $Na_2S_2O_3$ solution for 10 min.
2. The gel plugs were washed 3 x in 400 µL double distilled water for 10 min and the water was discarded.
3. 50 µL of NH_4HCO_3 buffer were added and the gel plugs were incubated for 10 min.
4. The gel plugs were destained for 10 min with 50 µL of a 1:1 (v/v) mixture of acetonitrile and NH_4HCO_3.
5. Proteins within the gel plugs were reduced in 50 µL of the dithiothreitol/NH_4HCO_3 solution for 15 min at 60 °C.
6. Proteins within the gel plugs were alkylated by incubation in 50 µL of the iodoacetamide/NH_4HCO_3 solution for 15 min.
7. The gel plugs were washed in 50 µL NH_4HCO_3 solution for 10 min.
8. The gel plugs were destained in 50 µL acetonitrile/NH_4HCO_3 solution for 10 min.
9. Steps 7. and 8. were repeated twice.
10. The gel plugs were dried in a vacuum centrifuge set to 45 °C.

11. 5 µL of trypsin working solution were added and allowed to be soaked into the gel. 10 µL NH_4HCO_3 buffer were added on top and the samples were incubated overnight. Peptides were released from the gels into the buffer by 10 min of ultrasound treatment in an ultrasound bath.

MALDI-TOF-MS and data acquisition:

1. 0.5 µL of sample were mixed with 0.5 µL matrix-solution. 2 x 0.5 µL were pipetted onto two MALDI targets and allowed to dry.
2. 0.5 µL of the calibration standard were applied onto the designated MALDI targets and allowed to dry.
3. MS-spectra were acquired with a MALDI-TOF/TOF-MS 4800 Proteomics Analyzer set to laser intensity of 2900 mW, 500 total shots per spectrum, mass range 800 – 4000 Da, mass focus 2000 Da, MS reflector positive, acquisition control automatic.
4. MS-data were processed with the AB SCIEX Data Explorer software.
5. Protein identification was carried out with the Mascot – peptide mass fingerprint software (Matrix Science; Perkins *et al.* 1999) using the NCBInr database, choosing trypsin as enzyme, carbamidomethyl (C) as fixed modification, peptide tolerance of ± 0.15 Da, MH^+ mass values and allowing up to 1 missed cleavage. Protein mass was given by the Mascot software.
6. Theoretical pI were determined with the help of the Compute pI/Mw software (ExPASy Bioinformatics Resource Portal; http://web.expasy.org/compute_pi/). Allocation of proteins was predicted using the CELLO v.2.5: subCELlular LOcalization predictor software (Molecular Bioinformatics Center, National Chiao Tung University; http://cello.life.nctu.edu.tw/).

Optional enrichment of peptides:

In case of low MS-signal intensities trypsin digested samples were enriched using ZipTip C18 pipette tips (Millipore). Prior to use the C18 bed was successively conditioned by 3 x aspirating and dispensing of 10 µL volumes of acetonitrile, followed by 3 x aspirating and dispensing 10 µL volumes of 50 % acetonitrile/0.1 % trifluoroacetic acid solution and 3 x aspirating and dispensing of 10 µL volumes 0.1 % trifluoroacetic acid. Tryptic digests were

loaded onto the C18 bed by aspirating and dispensing the sample (obtained in step 11 of sample clean-up and digestion) 10 times. The peptides were eluted by aspirating 2 µL of matrix solution and dispensing equal volumes (approximately 1 µL) onto two MALDI targets.

3.13 Gel staining and image acquisition

3.13.1 Coomassie Brilliant Blue staining

Gels were stained according to an improved Coomassie Brilliant Blue (CBB-G250) protocol developed by Kang *et al.* (2002). Proteins were fixed by incubating the gels in 500 mL 30 % ethanol containing 2 % phosphoric acid for 1 h. Gels were stained overnight in 500 mL staining solution composed of 0.02 % CBB-G250, 2 % (w/v) phosphoric acid, 5 % aluminum sulfate and 10 % ethanol. All staining steps were performed in plastic containers at slight shaking on a rocking platform at room temperature. Gels were scanned with the Imaging Densitometer GS-700 (Bio-Rad).

3.13.2 Ethidium bromide staining

Agarose gels were stained in a 1 L ethidium bromide bath (1 µg mL^{-1}) for 15 min, followed by destaining for up to 5 min in deionized water. The staining was carried out in containers with lid made of stainless steel. The gels were examined with the Molecular Imager Gel Doc, Universal Hood II (Bio-Rad).

3.13.3 Silver staining

Silver staining of gels was performed according to the protocol described by Blum *et al.* (1987) and shown in Tab. 3.6. 400 mL of each solution were prepared fresh in deionized water. All staining steps were performed in plastic containers at slight shaking on a rocking platform at room temperature. Gel images were taken with the Imaging Densitometer GS-700 (Bio-Rad).

Table 3.6: Silver staining protocol according to Blum *et al.* (1987).

Step	Solution	Time of treatment
Fix	50 % (v/v) methanol 12 % (v/v) acetic acid 0.5 mL L^{-1} 37 % formaldehyde	≥ 1 h
Wash	50 % (v/v) ethanol	3 x 20 min
Pretreat	0.2 g L^{-1} $Na_2S_2O_3$ x 5 H_2O	1 min
Rinse	Deionized water	3 x 20 min
Impregnate	2 g L^{-1} silver nitrate 0.75 mL L^{-1} 37 % formaldehyde	20 min
Rinse	Deionized water	2 x 20 min
Develop	50 g L^{-1} Na_2CO_3 0.5 mL L^{-1} 37 % formaldehyde 4 mg L^{-1} $Na_2S_2O_3$ x 5 H_2O	1 – 10 min
Wash	Deionized water	2 x 2 min
Stop	50 % (v/v) methanol 12 % (v/v) acetic acid	10 min
Wash	50 % (v/v) methanol	≥ 20 min

3.13.4 Simply Blue Safe Stain

Zymogram gels were stained with the commercially available Simply Blue Safe Stain (Invitrogen/Molecular Probes) according to the manufacturer's instructions. Gels were washed in 100 mL deionized water 3 x for 5 min, discarding the water after each interval. The gels were covered with the staining solution and incubated for 1 h at room temperature. Gels were washed 2 x for 1 h in deionized water. All staining steps were performed in plastic containers at gentle agitation on a rocking platform at room temperature. Gel images were taken with the Imaging Densitometer GS-700 (Bio-Rad).

3.13.5 SybrGold staining

Fluorescent staining of DGGE gels was performed with the commercially available SybrGold (Invitrogen/Molecular Probes) stain. The reagents were prepared according to the manufacturer's instructions. Gels were stained for 1 h in 500 mL 1 x TAE-buffer (Bio-Rad) adjusted to pH 8.0 and containing 50 µL of the 10,000 x SybrGold concentrate. The staining

was performed in a lightproof, plastic staining container with lid, placed on a rocking platform which was set to gentle agitation. Gels were scanned with a Molecular Imager FX pro plus Fluorescence Laser Scanner (Bio-Rad) connected to an FX External Laser Module (Bio-Rad). Excitation was set to 488 nm and fluorescence was detected using a 530 nm band pass filter.

3.13.6 SyproRuby protein gel stain

SDS-Tris-glycine gels were stained with the fluorescent dye SyproRuby (Invitrogen) according to the manufacturer's basic protocol instructions. Gels were incubated twice in 500 mL fixation solution (50 % (v/v) methanol, 7 % (v/v) acetic acid) for 30 min. The fixation solution was decanted and 500 mL of SyproRuby stain were added. The gels were incubated overnight in lightproof, plastic staining containers with lid. The gels were washed in 500 mL wash solution (10 % (v/v) methanol, 7 % (v/v) acetic acid) for 30 min and rinsed twice in deionized water for 5 min. All steps were performed on a rocking platform set to gentle agitation. Images were acquired with the Molecular Imager FX pro plus Fluorescence Laser Scanner (Bio-Rad) connected to an FX External Laser Module (Bio-Rad) set to excitation at 488 nm and emission detected at 610 nm.

4. RESULTS

The main aim of the current study was the characterization of drinking-water biofilms and their EPS grown in drinking water of different distribution systems as well as plumbing systems by microbiological, molecular biological and biochemical means. The first part of the study required establishment and adaptation of methods for the cultivation of drinking-water biofilms, for EPS isolation, as well as methods for quantitative and qualitative analyses of the biofilms and their EPS.

4.1 Establishment of methods for the cultivation of drinking-water biofilms and EPS recovery

4.1.1 Cultivation of drinking-water biofilms

Drinking-water biofilms were grown on an EPDM rubber material, which complied with physical and chemical requirements for rubber materials used in drinking water systems, but not with microbiological recommendations according to DVGW Code of Practice W270 (DVGW, 2007). In previous studies Bressler *et al.* (2009) showed that exposure of this EPDM material to drinking water resulted in significant growth of drinking-water biofilms. In their study the biofilms reached a quasi-stationary state after 14 d of incubation in flow-through reactors, and remained constant during further incubation for up to 5 weeks. The experimental setup by Bressler *et al.* (2009) was chosen as basis for the current study.

EPDM coupons were inserted into stainless-steel flow-through reactors, which were connected to drinking water taps of plumbing system A and continuously perfused with 50 mL min^{-1} drinking water for 14 d. The mean water temperature was 17.3 °C ± 2.9 °C

(range 10.8 °C to 21.4 °C; n = 30 over 5 independent reactor runs). Free chlorine was usually below the limit of detection (< 0.01 mg L^{-1}) with occasional readings of 0.01 mg L^{-1}. The 14 d cultivation of drinking-water biofilms on EPDM in this study resulted in macroscopically visible, multi-layered biofilms with a biofilm thickness of approximately 100 µm estimated by CLSM (Fig. 4.1). The wet weight was 11.3 ± 3.6 mg cm^{-2}, the dry weight was 0.09 ± 0.03 mg cm^{-2}, and the water content was 99.2 ± 0.2 % (n = 4 independent reactor runs). SEM and CLSM images of 14 d-old biofilms indicated a relatively dense coverage of the rubber material by biofilm cells. Total cell counts were 2.6 x 10^8 ± 1.4 x 10^8 cells cm^{-2} (n = 10 independent reactor runs). The culturability of 14 d-old biofilm cells was approximately one magnitude lower compared to the cell counts, showing HPC values of 3.1 x 10^7 ± 2.5 x 10^7 cfu cm^{-2} (n = 10 independent reactor runs).

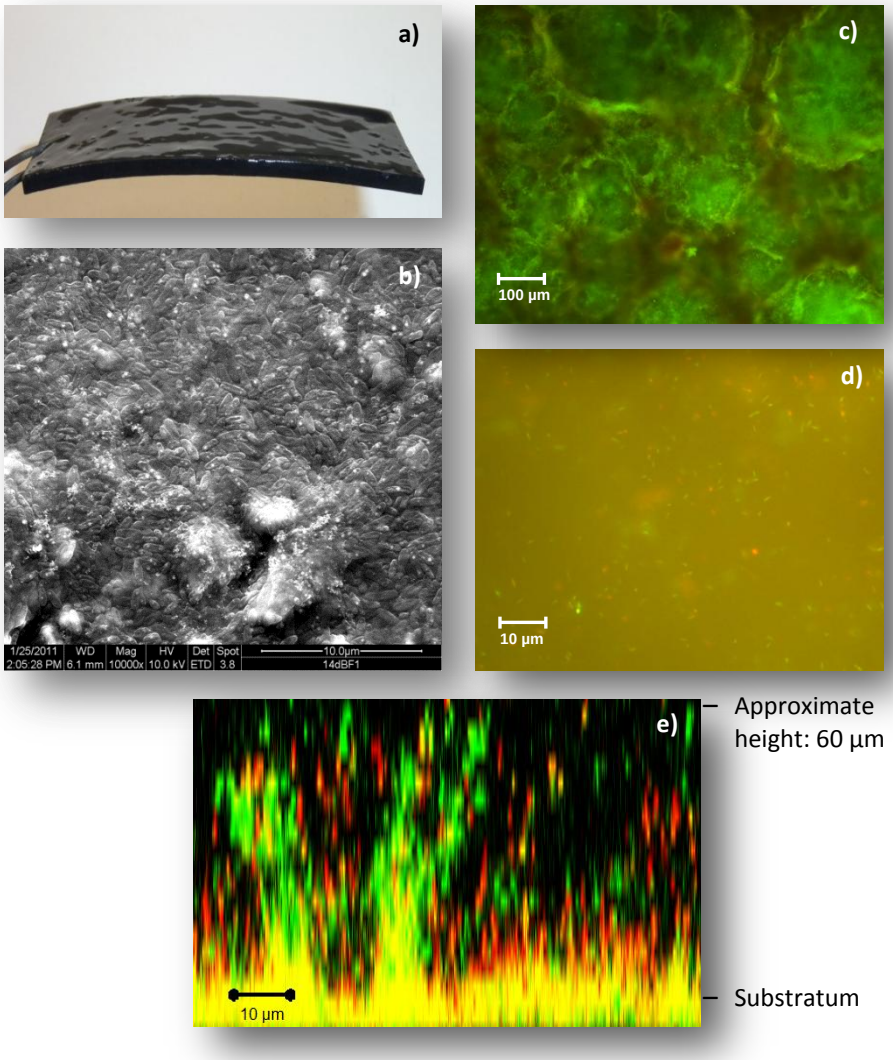

Figure 4.1: Images of 14 d-old drinking-water biofilms grown on EPDM showing a) a macroscopical view of the biofilm, b) an SEM top view of a dried biofilm at 10,000 x magnification, c) and d) epifluorescence microscopic top views of live/dead stained biofilms at 100 x and 1,000 x magnification, respectively and e) a CLSM side-view (z-stack) image of the lower 60 µm of a live/dead stained biofilm at 1,000 x magnification.

4.1.2 Optimization of EPS isolation by CER

The CER method for isolation of EPS was adapted from Jahn and Nielsen (1995), who isolated EPS from sewer biofilms and pure culture biofilms of *P. putida* on scales of several hundred mL. This method functions by removal of multivalent cations, which cross-link polymers within the biofilm matrix, and consequential destabilization of the EPS matrix. The CER isolation required miniaturization and optimization to meet the requirements of the usually low quantities of EPS in drinking-water biofilms. The duration of CER treatment was optimized with respect to the yield of EPS components and detrimental effect on biofilm organisms. 8 mL aliquots of biofilm suspensions (corresponding to approximately 8×10^8 cells mL^{-1}) were shaken on a Vortex shaker in 50 mL centrifuge tubes for up to 60 min at room temperature with or without CER to determine optimal treatment duration. Proteins, carbohydrates and DNA were quantified in filter sterilized (pore size 0.2 µm) undialysed EPS solutions.

Proteins, carbohydrates and DNA were present in quantifiable concentrations in EPS fractions isolated by shaking without or with CER from 14 d-old drinking-water biofilms. Presence of uronic acids was not detected. Significantly increased yields of all measured EPS components compared to simple shaking treatment were found in EPS isolated from drinking-water biofilms with the help of the CER ($p < 0.05$) (Fig. 4.2). For carbohydrates and proteins the most significant increase in recovery occurred within 20 min of CER treatment. For this duration the yields of carbohydrates and proteins were increased 2.2 fold (2.25 µg cm^{-2}) and 3.5 fold (3.66 µg cm^{-2}), respectively. Prolonged CER isolation of 40 min or 60 min showed no statistically significant further increase in protein or carbohydrate recovery compared to CER treatment for 20 min ($p > 0.05$). The DNA yield was 146 fold (0.29 µg cm^{-2}) increased by 20 min CER isolation compared to shaking without CER. Prolongation of the CER isolation procedure resulted in further increase of DNA recovery to concentrations of 0.6 µg cm^{-2} after 60 min of CER treatment.

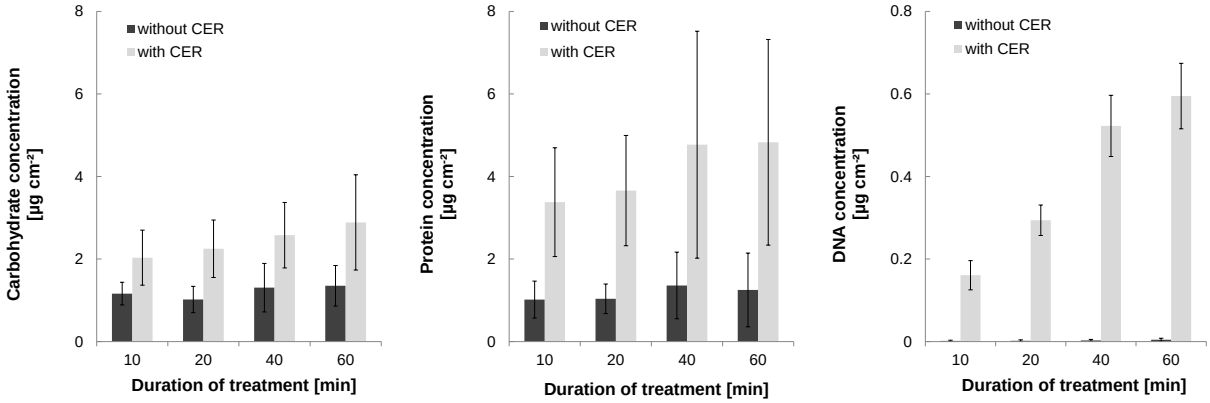

Figure 4.2: Protein, carbohydrate and DNA concentrations in EPS of 14 d-old drinking-water biofilms after EPS isolation by shaking without (dark bars) or with (light bars) the use of CER. Proteins were quantified by the modified Lowry assay, carbohydrates were determined by the sulphuric acid/phenol method, eDNA was analyzed by PicoGreen (n = 4 independent reactor runs).

4.1.3 Cell integrity measurements after CER isolation

Potential lysis of drinking-water biofilm cells induced by the CER isolation procedure was analyzed by determining culturability of cells after EPS isolation and resuspended in the initial volume of 6 mM phosphate buffer (pH 7.0), presence of the strictly intracellular enzyme G6PDH in the EPS, as well as presence of the LPS component KDO in the EPS.

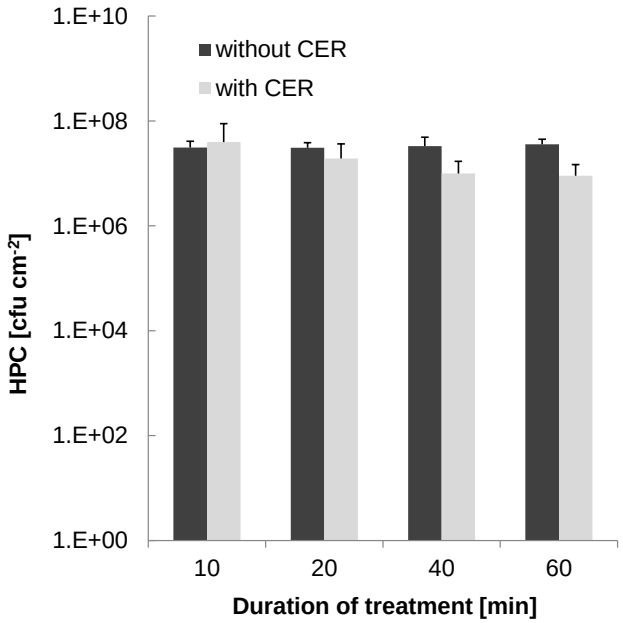

Figure 4.3: Culturability of 14 d-old drinking-water biofilm cells after shaking treatment without (dark grey bars; n = 2) or with (light grey bars; n = 4) CER for up to 60 min. HPC was determined by spread-plate method on R2A medium and enumeration of colonies after incubation for 7 d at 20 °C.

Total cell counts and culturability of biofilm cells was determined for the initial biofilm suspension, as well as biofilm cells after EPS isolation by shaking in presence or absence of CER for up to 60 min. Total cell counts showed similar values for the biofilm suspension as well as biofilm cells after EPS isolation by shaking or CER, which were in the range of 2.6 x 10^8 cells cm^{-2} and 4.4 x 10^8 cells cm^{-2}. The biofilm cells showed no statistically significant decrease of culturability due to the EPS isolation procedure in presence or absence of CER (Fig. 4.3). A slightly decreasing trend of culturability, though not being of statistical significance, could be noticed with prolonged CER treatment from initially 4.2 x 10^7 cfu cm^{-2} in the untreated biofilm suspension down to 9.0 x 10^6 cfu cm^{-2} after 60 min of treatment.

The activity of G6PDH in the EPS solution prepared by shaking treatment for 20 min or 60 min in presence or absence of CER was applied as further means to detect cell lysis caused by CER treatment. Measurements of G6PDH activity in cell extracts confirmed the presence of the enzyme within drinking-water biofilm cells, showing a specific activity of 0.03 ΔA min^{-1} mg^{-1} protein (Fig. 4.4). Activity of G6PDH in the EPS solutions isolated without or with CER could not be detected, regardless of the duration of CER treatment, suggesting that significant lysis due to shaking in absence or in presence of CER did not occur.

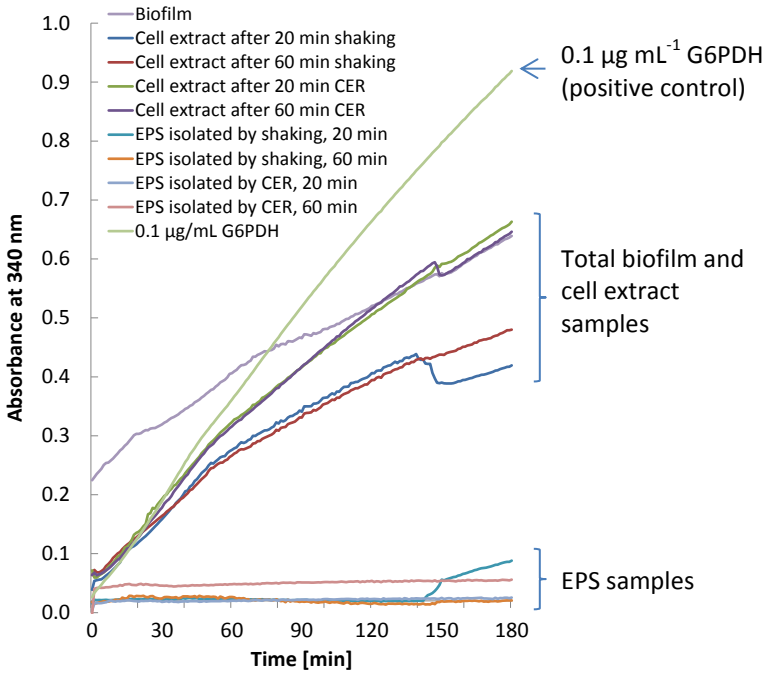

Figure 4.4: G6PDH activity within concentrated total biofilm, as well as cell extracts and EPS obtained by 20 and 60 min of EPS isolation treatment by shaking and CER and concentrated 20 fold.

KDO is a characteristic component of LPS in the outer membrane of Gram-negative bacteria and was additionally applied as means to determine cell damage. KDO could not be detected in the EPS, nor in the initial biofilm suspension, which was used as positive control of presence of KDO in the biofilms. This indicated that the sensitivity of the KDO assay is insufficient for determination of KDO in the EPS of drinking-water biofilms.

20 min shaking in combination with CER was set as standard for further analyses, showing significantly higher EPS yields compared to shaking treatment without CER and no noticeable impact on biofilm cells.

4.1.4 Efficiency of CER isolation

The efficiency of EPS isolation by CER is attributed to its capability to bind and in this way remove multivalent cations such as Ca^{2+}, Mg^{2+}, $Fe^{2+/3+}$ or $Cu^{1+/2+}$ from the biofilm suspension, which in their presence interact with negatively charged sites of EPS components causing bridging of polymers (Park and Novak, 2007). Removal of these cations results in the destabilization of the EPS matrix and increased solubilization of EPS components. The presence of $Ca^{2+}$and Mg^{2+}, which are characteristic cations in drinking water, and $Fe^{2+/3+}$ and $Cu^{1+/2+}$, which are most frequently used in materials in drinking water distribution systems, was confirmed in drinking-water biofilms by SEM imaging coupled with an energy-dispersive x-ray spectroscopic microanalysis (EDX; EDAX EDS-Analysis system, Genesis 4000). SEM images showed particulate deposits on or in between biofilm cells on the EPDM. EDX spectra identified Ca, Mg, Fe and Cu along with Zn and Si as most prominent cations in these deposits (Fig. 4.5, target), as well as throughout the drinking-water biofilm matrix.

The metal cations were quantified in drinking water and in biofilm samples by ICP-OES and the concentrations were compared on a wet weight basis, assuming the wet weight of 1 mL of drinking water to be 1 g. The quantification of the cations in drinking-water biofilms revealed a 1.9 fold accumulation Ca^{2+}, a 2.7 fold accumulation of Mg^{2+}, and a 500 fold accumulation of $Fe^{2+/3+}$ and $Cu^{1+/2+}$compared to the water phase (Tab. 4.1).

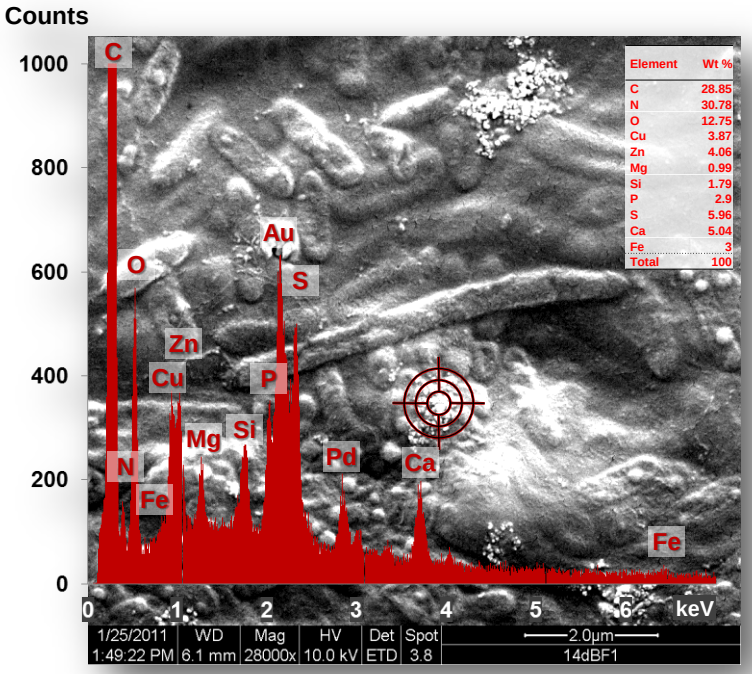

Figure 4.5: SEM image including an EDX spectrum and relative composition of inorganic deposits found in a dried 14 d-old drinking-water biofilm. Target indicates the region analyzed by the EDX-probe.

The efficiency of the CER to bind and in this way to remove multivalent cations from the biofilm suspension was analyzed by determining the concentrations of Ca^{2+}, Mg^{2+}, $Fe^{2+/3+}$ and $Cu^{1+/2+}$ in different biofilm fractions in the course of EPS isolation by CER. The fractions were the initial biofilm suspensions of 14 d-old drinking-water biofilms, the biofilm suspensions treated for 20 min with CER, as well as the EPS solutions obtained after 20 min CER treatment, centrifugation and filter sterilization. The quantification allowed for calculation of the proportions of the metals associated with the cells (including metals strongly bound to or within cells, or in particulate form), those remaining in the EPS, or the proportions of the metals removed by the CER (Tab. 4.1). Ca^{2+} was removed by > 86.3 %, $Fe^{2+/3+}$ by 36.7 ± 31.6 % and $Cu^{1+/2+}$ by 43.9 ± 1.8 % from the biofilm suspension by using CER. The concentration of Mg^{2+} in the CER treated biofilm suspension decreased with over 56.3 % below limit of detection of the applied method. $Fe^{2+/3+}$ and $Cu^{1+/2+}$ were additionally removed by 56.5 ± 27.4 % and 52.0 ± 1.5 % in the course of EPS clean-up by centrifugation and filter sterilization, respectively, suggesting that a high proportion of these cations was bound to the cell material, present within biofilm cells, or in particulate form.

Table 4.1: Comparison of metal ion concentrations in drinking water and in 14 d-old drinking-water biofilm, as well as the distribution of the cations after EPS isolation by 20 min CER treatment. 100 % correspond to concentrations in the biofilm suspension. Metal concentrations were determined by ICP-OES (n = 2 independent reactor runs); n. d., not detected.

Cation	Concentration		Distribution of cations after EPS isolation		
	Drinking water	Biofilm	Cells	EPS	CER
	[µg mL^{-1}]	[µg g^{-1} wet weight]	[%]		
Ca^{2+}	109 ± 7.1	205.8 ± 67.4	n. d.	7.6 ± 1.7	> 86.3
Mg^{2+}	11.4 ± 0.7	30.2 ± 9.9	n. d.	n. d.	> 56.3
$Fe^{2+/3+}$	< 0.015	7.4 ± 3.0	56.5 ± 27.4	6.8 ± 4.5	36.7 ± 31.6
$Cu^{1+/2+}$	0.087 ± 0.001	47.7 ± 1.6	52.0 ± 1.5	4.1 ± 0.3	43.9 ± 1.8

4.2 Optimization of 2D gel electrophoresis for EPS proteins

2D gel electrophoresis (2DE) was applied to qualitatively analyse EPS proteins from drinking-water biofilms and to be able to compare different EPS isolation techniques on a qualitative level. 2DE required additional sample preparatory steps and an optimization of the isoelectric focusing step in order to produce well resolved protein spot patterns of EPS proteins.

4.2.1 EPS sample preparation for 2DE

EPS isolated from 14 d-old drinking-water biofilms by 20 min CER treatment were used for the optimization of 2D gel electrophoresis for EPS proteins. In a first attempt the EPS solutions recovered by 20 min CER isolation, centrifugation and filter sterilization (pore size 0.2 µm) (Section 3.8) were freeze-dried directly without any additional clean-up steps, resuspended in IEF buffer and subjected to 2DE. IEF was carried out for 75 kVh. Gels were stained by silver (Blum *et al.*, 1984).

2DE of EPS samples without further clean-up resulted in a low number of distinct spots (< 50) most of which were located in the acidic pH range. However, the gels showed a smear and vertical streaking throughout the gel and a clearing zone in the region between pH 8 and pH 9 appeared, which indicated interference of inorganic salts or EPS components other than proteins during the focusing step of the 2DE (Fig. 4.6a).

To eliminate interference of salts during IEF, EPS samples were dialyzed against deionized water prior to freeze-drying and applied to 2DE. Dialysis of EPS samples allowed for detection of a larger diversity of proteins by 2DE compared to undialyzed samples, showing up to 100 focused spots located mainly in the acidic region between pH 3 and pH 7 (Fig. 4.6b). The clearing zone could be reduced, however, vertical streaking was still present, especially in regions between pH 5 and pH 8, impeding the evaluation of the gels.

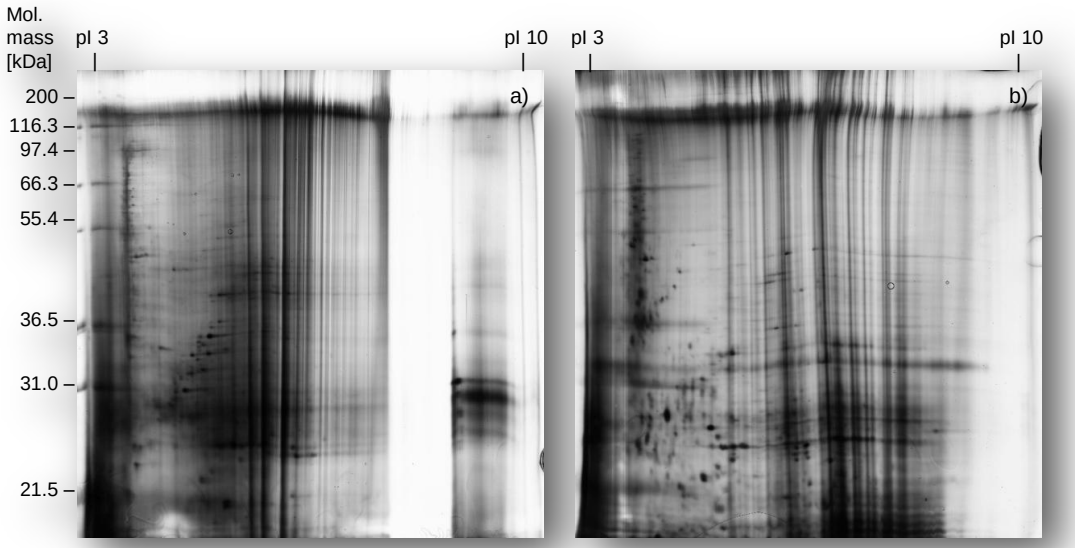

Figure 4.6: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment a) without any sample clean-up and b) after dialysis. Protein load: 200 µg.

DNA and certain carbohydrates are known to interfere with 2DE by clogging pores of IEF gels or directly by interacting with proteins, rendering their charge or size (Garfin and Heerdt, 2000). Extracellular DNA and carbohydrates were present in the isolated EPS of 14 d-old drinking-water biofilms at considerable concentrations of 1.1 ± 0.1 µg mL^{-1} and 8.4 ± 2.6 µg mL^{-1} (Section 4.1.2), respectively, and therefore, could cause interferences during 2DE. DNA was removed from filter-sterilized EPS solutions by incubation with 65 U mL^{-1} DNase Benzonase for 1 h at 37 °C. Preliminary experiments indicated that these conditions resulted in degradation of DNA by > 99.5 %. DNA-free EPS solutions were dialyzed to remove interfering salts, freeze-dried and applied to 2DE.

Removal of DNA from EPS samples and subsequent dialysis resulted in well-resolved spots throughout all pH regions and with no noticeable interference. Usually, a total number ranging between 600 to 800 spots could be found in the EPS of 14 d-old drinking-water biofilms after clean-up with DNase and dialysis. The majority of spots were detected

between pH 4 and pH 8 and molecular weightes of 21.5 kDa and 100 kDa. In one case a total number of up to 1400 spots could be resolved in pH regions between pH 3 and 10 and molecular weightes between 21.5 kDa and 200 kDa (Fig. 4.7). Recovery of a total of 1400 spots, however, was achieved only once. DNase treatment with subsequent dialysis of EPS samples were implemented in the sample clean-up for further analyses by 2DE.

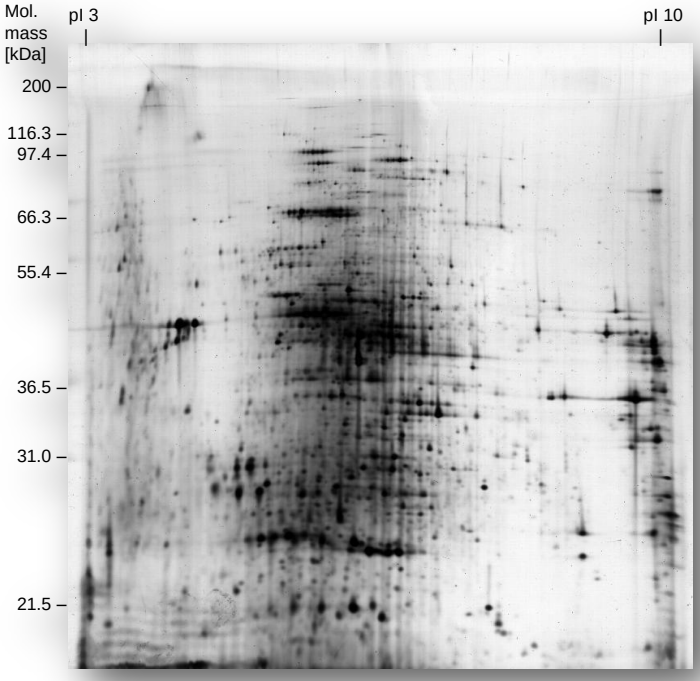

Figure 4.7: 2DE gel of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment optimized by EPS clean-up with DNase (Benzonase) treatment and subsequent dialysis. Protein load: 200 µg.

4.2.2 Optimization of isoelectric focusing (IEF) for EPS samples

IEF of EPS proteins isolated from 14 d-old drinking-water biofilms was optimized with regard to focusing duration, to produce high resolved protein spots in preferably short periods. IEF was carried out for a total of 45 kVh to 95 kVh with 10 kVh increments.

Focusing for a total of 45 kVh resulted in well resolved spots in the neutral and acidic regions of the gel, however, the basic region exhibited pronounced horizontal streaking indicating incomplete focusing of proteins (Fig. 4.8). Prolonged focusing for 55 kVh for the most part eliminated horizontal streaking in the basic region, showing only a small number of blurred spots. IEF carried out for ≥ 65 kVh generally produced well resolved spots and similar protein patterns without noticeable interferences. 75 kVh were used for further analyses by 2DE to ensure sufficient focussing.

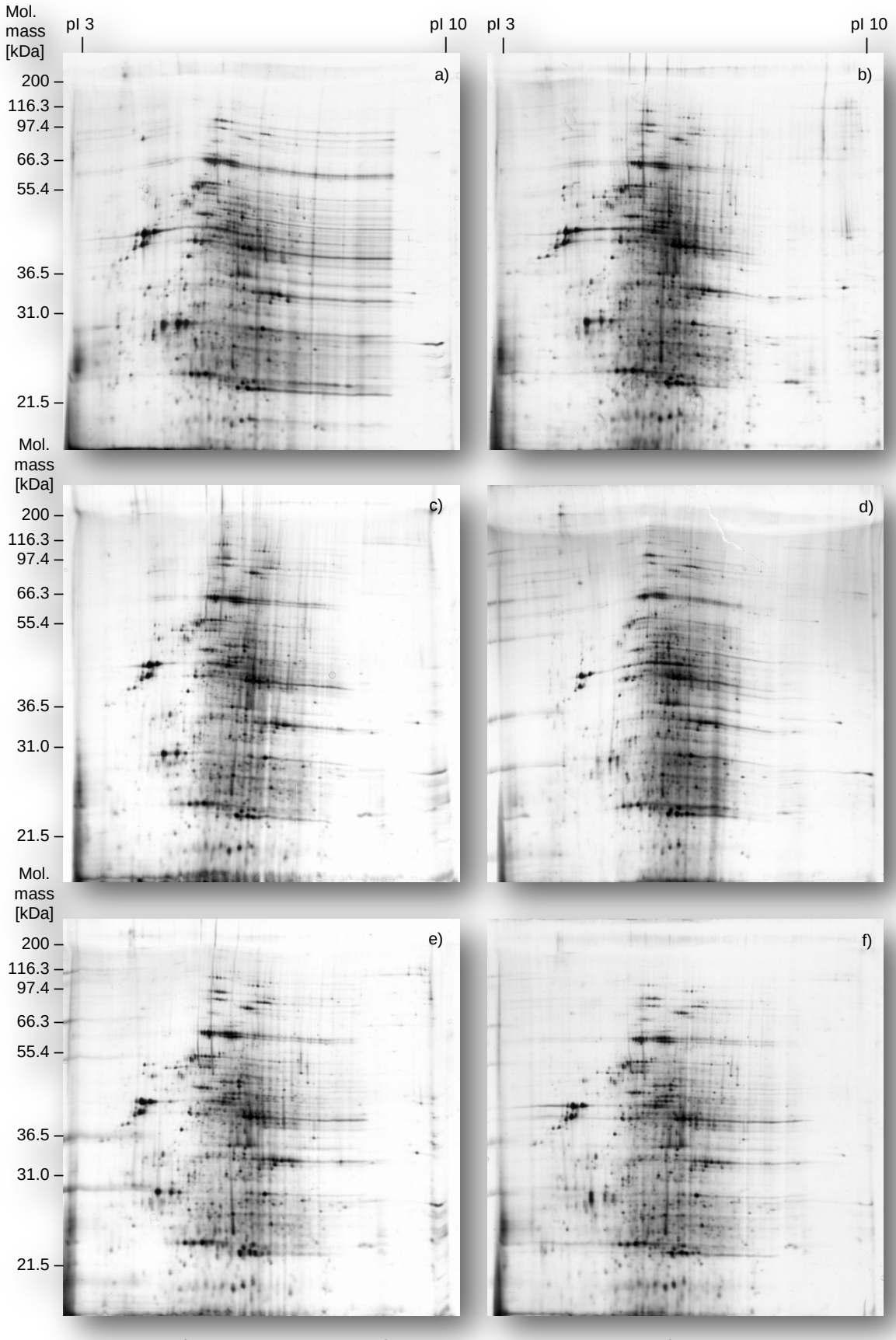

Figure 4.8: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by 20 min CER treatment and focused for a) 45 kVh, b) 55 kVh, c) 65 kVh, d) 75 kVh, e) 85 kVh or f) 95 kVh. Protein load: 200 µg.

4.2.3 Selection of staining method for SDS-Tris-glycine gels

Three staining protocols were tested and compared for their capability to stain EPS proteins from 14 d-old drinking-water biofilms, which were separated by 2DE on large format (20 cm x 20 cm x 0.1 cm) SDS-Tris-glycine gels. The methods were silver staining as described by Blum *et al.* (1984), an improved Coomassie brilliant blue staining technique according to Kang *et al.* (2002), and the commercially available SyproRuby protein gel stain (Invitrogen/Molecular Probes) (Section 3.13).

The comparison showed that silver staining according to Blum *et al.* (1984) was the most sensitive and, therefore, most suitable method to stain gels containing isolated EPS proteins (Fig. 4.9c). The improved Coomassie brilliant blue staining, which according to Kang *et al.* (2002) is supposed to be as sensitive as silver staining when using mini-gels (7 cm x 7 cm x 0.1 cm), in this study only showed a total of 10 weakly stained spots on the large format gels (20 cm x 20 cm x 0.1 cm) (Fig. 4.9a). Also the bands of the protein marker Mark12, applied on the gel for molecular weight estimation of the proteins, appeared very faint, indicating that the protocol by Kang *et al.* (2002) is not adequate to stain EPS proteins on large format gels as applied in this study. The fluorescent SyproRuby stain, which according to the manufacturer (Invitrogen/Molecular Probes) is also supposed to be as sensitive as silver staining, showed a total of 221 spots, however, the fluorescence signal was relatively weak and the diversity of detected spots was lower compared to silver staining (Fig. 4.9b). Silver staining according to Blum *et al.* (1984) was used for further staining of 2DE gels.

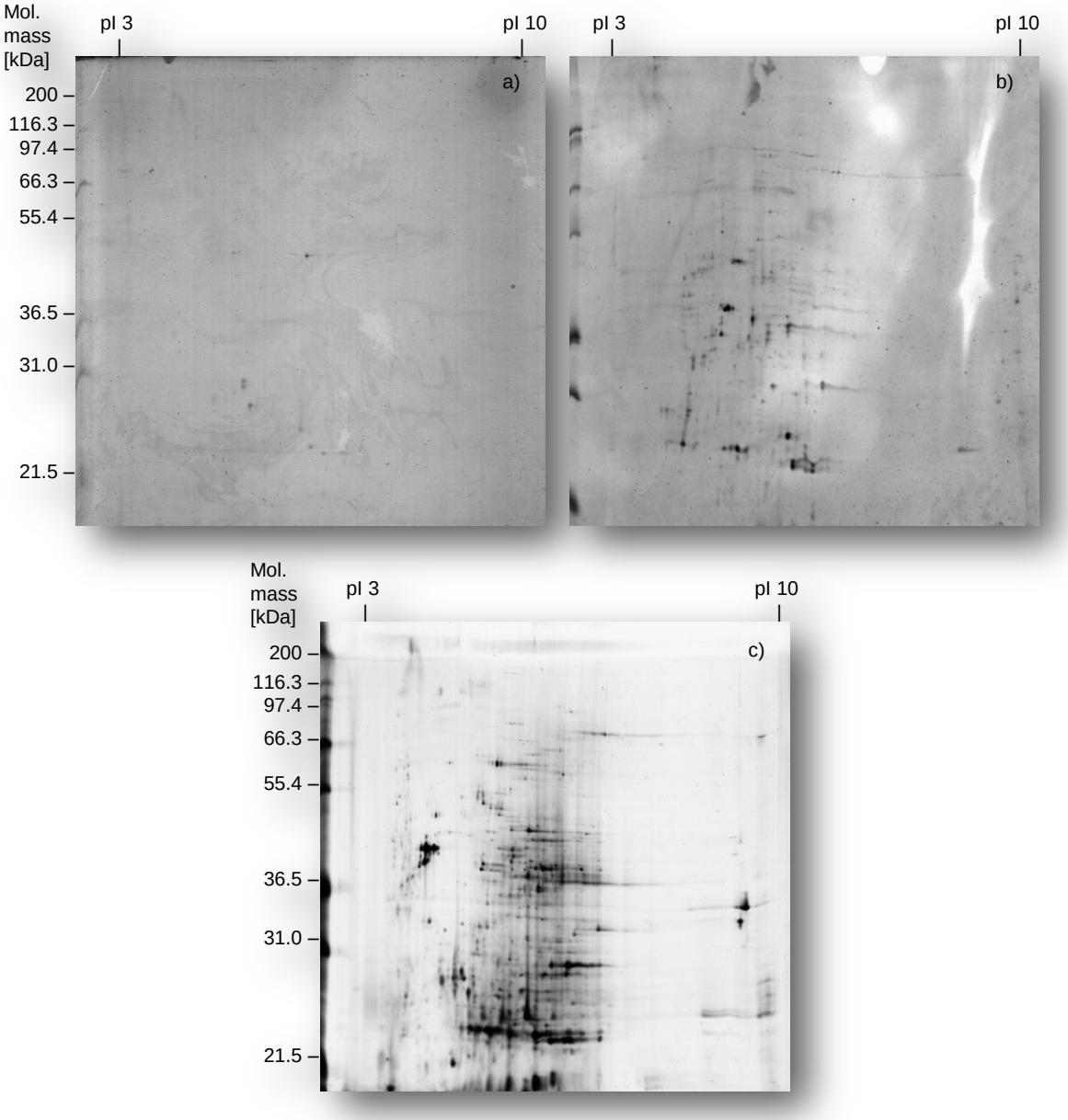

Figure 4.9: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms isolated by 20 min CER treatment and stained with a) Coomassie brilliant blue (Kang *et al.*, 2002), b) SyproRuby protein gel stain or c) silver (Blum *et al.*, 1984). Protein load: 200 µg.

4.3 Comparison of EPS isolation methods

The optimized CER isolation was compared to alternative EPS isolation methods by NaOH in combination with formaldehyde, EDTA, or heat treatment in order to evaluate the effectiveness of the CER method. The methods were evaluated by the yields of isolated EPS determined quantitatively by photometric or fluorometric methods or qualitatively by 2D gel electrophoresis. Furthermore, the detrimental impact on biofilm cells, as well as the potential interference of these isolation methods with subsequent analytical methods was ubvestugated.

4.3.1 Quantitative comparison of EPS recovery

Drinking-water biofilm suspensions, biofilm cells after EPS isolation (cells), and isolated EPS were analyzed for protein, carbohydrate and DNA concentrations to evaluate the efficiency of the EPS isolation techniques on a quantitative level. Analysis of total biofilm suspension revealed overall protein concentrations of 33.9 ± 5.6 µg cm^{-2} and carbohydrate concentrations of 6.1 ± 2.1 µg cm^{-2} within the biofilm. All EPS isolation methods provided quantifiable amounts of EPS from drinking-water biofilms (Fig. 4.10). Proteins represented the main component of the EPS, followed by carbohydrates and DNA. Isolation by CER, formaldehyde/NaOH, EDTA and heat showed increased yields of all measured EPS components compared to the reference treatment by shaking.

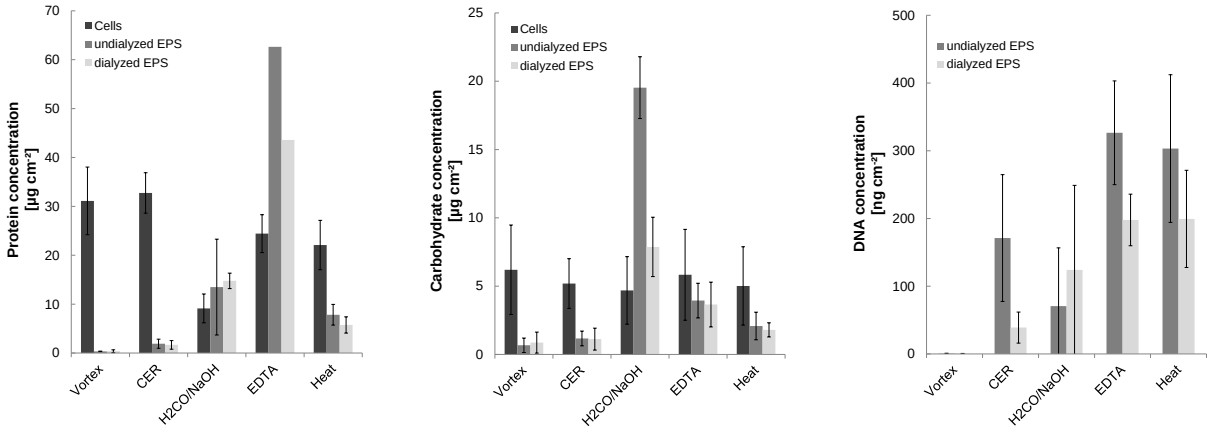

Figure 4.10: Protein, carbohydrate and DNA concentrations in the cell fraction after EPS isolation (cells) and EPS isolated from 14 d-old drinking-water biofilms by shaking (Vortex), CER, formaldehyde/NaOH, EDTA or heat. Proteins were quantified by the modified Lowry assay (Peterson, 1977), carbohydrates were determined by the sulphuric acid/phenol method, DNA was analyzed by PicoGreen (n = 3 independent reactor runs).

In this set of experiments CER treatment resulted in a protein yield of 1.9 ± 0.9 μg cm^{-2}, a carbohydrate yield of 1.2 ± 0.5 μg cm^{-2} and a DNA yield of 0.17 ± 0.09 μg cm^{-2}. However, this method seemed least effective in extracting proteins and carbohydrates compared to the other methods. Interference of the CER with subsequent analyses was not observed.

Formaldehyde/NaOH treatment to isolate EPS from drinking-water biofilms resulted in highest protein yields (13.5 ± 9.8 μg cm^{-2}), but lowest DNA yields (70.7 ± 86.0 μg cm^{-2}). The sulphuric acid/phenol method for quantitation of carbohydrates revealed carbohydrate concentration of 19.5 ± 2.3 μg cm^{-2} in the EPS, which was more than three times the amount of carbohydrates determined for the total biofilm (6.1 ± 2.1 μg cm^{-2}), indicating interference of the chemicals with the quantification. Dialysis of the EPS solutions against deionized water decreased carbohydrate concentrations, however, concentrations in the EPS still exhibited higher contents than the total biofilm sample (Fig. 4.10). In order to identify the source of interference when using formaldehyde/NaOH with the sulphuric acid/phenol method, blank samples containing deionized water only were treated according to the isolation procedure (Section 3.8), or separately with formaldehyde or NaOH. Blank experiments revealed a significant yellow discoloration of the blank solution in presence of formaldehyde after addition of sulphuric acid and phenol, showing an absorbance equivalent to carbohydrate concentration of 48.9 ± 10.6 μg mL^{-1}, despite absence of carbohydrates (Fig. 4.11). This effect was increased when formaldehyde was applied in combination with NaOH in the blank solutions, showing an absorbance equivalent to 67.1 ± 8.3 μg mL^{-1}. Addition of NaOH alone to deionized water resulted in only slightly elevated absorbance during carbohydrate quantitation, equivalent to 1.9 ± 1.0 μg mL^{-1}. Hence, formaldehyde was the main agent causing interference with the sulphuric acid/phenol method. Protein quantification was also obstructed by the presence of NaOH. Blank samples containing deionized water and NaOH showed slightly decreased absorbance values compared to deionized water without NaOH, while formaldehyde showed no effect on the modified Lowry measurement. The presence of NaOH, therefore, results in an underestimation of protein concentrations. Quantitation of DNA in the blank samples treated with formaldehyde/NaOH indicated no interference of formaldehyde, NaOH or their combination with the PicoGreen assay. However, analysis of EPS isolated from drinking-water biofilms by

formaldehyde/NaOH showed higher DNA concentrations once the samples were dialyzed, compared to undialyzed samples.

The use of EDTA to isolate EPS showed highest yields of carbohydrates (3.7 ± 1.6 μg cm^{-2}) and DNA (0.33 ± 0.08 μg cm^{-2}) (Fig. 4.10). An exact determination of isolated proteins by EDTA with the modified Lowry assay was disturbed by the formation of a precipitate after addition of the Folin Ciocalteu's phenol reagent, irrespective of the sample type (isolated EPS or blank). The resulting absorbance, equivalent to a protein concentration of 62.6 μg cm^{-2}, by far exceeded overall protein concentration in the total biofilm (33.9 ± 5.6 μg cm^{-2}). Dialysis of EPS isolated by EDTA against deionized water resulted in decreased absorbance values when performing protein quantification, corresponding to protein concentrations of 43.6 μg cm^{-2}, which were still higher than total protein content in the biofilm. Moreover, the presence of EDTA in the sample caused an increase in sample volume during dialysis, which was approximately 2 fold higher compared to undialysed samples. Consequentially analytes were 2 fold diluted. The interference of EDTA during protein quantification was backed up by blank experiments, applying deionized water as sample for EPS isolation with EDTA (Section 3.8). Presence of EDTA strongly interfered with the Lowry assay, resulting in an absorbance equivalent to protein concentrations of 320 ± 325 μg mL^{-1} despite the absence of proteins (Fig. 4.11). The quantification of carbohydrates in the EPS isolated by EDTA using the sulphuric acid/phenol method was also disturbed. The obtained values of carbohydrate concentration in the isolated EPS (3.7 ± 1.6 μg cm^{-2}) together with the concentration in the cell fraction after EPS isolation (5.8 ± 3.3 μg cm^{-2}) exceeded the total carbohydrate concentration in the biofilm (6.1 ± 2.1 μg cm^{-2}). There was no indication of interference of EDTA with the DNA quantification using PicoGreen, showing DNA concentrations of 327 ± 77 μg cm^{-2}.

Heat treatment was significantly more effective in isolating proteins (7.8 ± 2.1 μg cm^{-2}) and carbohydrates (2.1 ± 1.0 μg cm^{-2}) from drinking-water biofilms compared to CER treatment, and isolated DNA (303 ± 109 μg cm^{-2}) in concentrations similar to those obtained by EDTA isolation. Interference of heat treatment with subsequent analyses was not observed.

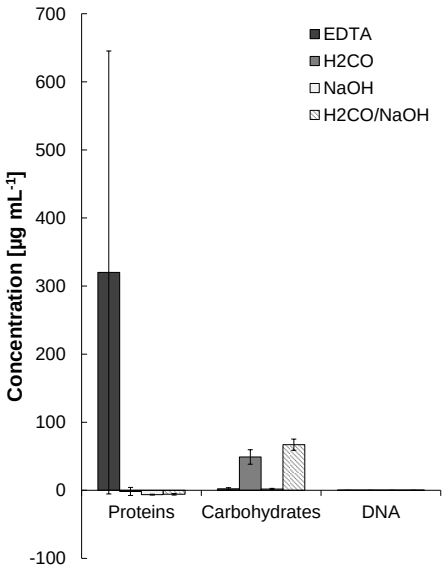

Figure 4.11: Protein, carbohydrate and DNA concentrations measured in deionized water after addition of EDTA, formaldehyde, NaOH or formaldehyde with NaOH. Proteins were quantified by the modified Lowry assay (Peterson, 1977), carbohydrates were determined by the sulphuric acid/phenol method, eDNA was analyzed by PicoGreen (n = 2 independent measurements).

To determine accuracy of the quantitation assays for the analysis of EPS from drinking-water biofilms and recovery rates of proteins, carbohydrates and DNA after each EPS isolation procedure, a model EPS solution containing BSA (10 µg mL^{-1}), dextran (5 µg mL^{-1}) and DNA (1 µg mL^{-1}) at similar concentrations and ratios as determined in the EPS of drinking-water biofilms was prepared in deionized water. The model EPS solution was subjected to the EPS isolation procedures by shaking (control), or treatments by CER, formaldehyde, NaOH, formaldehyde in combination with NaOH, EDTA or heat.

EPS isolation by shaking (control), CER or heat resulted in protein and carbohydrate recovery close to 100 % of the initial concentrations of the model EPS solution (Fig. 4.12). DNA was recovered at lower concentrations after treatment by shaking, CER and heat, showing 57 %, 84 % and 60 % of the initial concentration, respectively. Dialysis of the EPS solutions isolated by shaking or CER treatment resulted in a loss of protein by approximately 43 %, carbohydrates and DNA were only marginally reduced by dialysis.

Exposure of the model EPS solution to EDTA, formaldehyde, NaOH or formaldehyde in combination with NaOH resulted in similar interferences of subsequent quantitation methods as described before for EPS isolated from drinking-water biofilms and blank

samples (deionized water) (Fig. 4.12). EDTA caused values 30 times higher for proteins, 1.9 times higher for carbohydrates and 1.5 times higher for DNA than prepared in the model EPS. Samples treated with formaldehyde showed a recovery of only 25 % of proteins, 860 % of carbohydrates, or 29 % of DNA present in the model EPS. NaOH or formaldehyde/NaOH exposed model EPS showed no presence of proteins, while carbohydrate concentration was 2.6 fold and 13.7 fold higher, respectively, and DNA concentration was 85 fold or 87 fold lower, respectively. Dialysis of the model EPS against deionized water could to some extent reduce interfering effects of the chemicals.

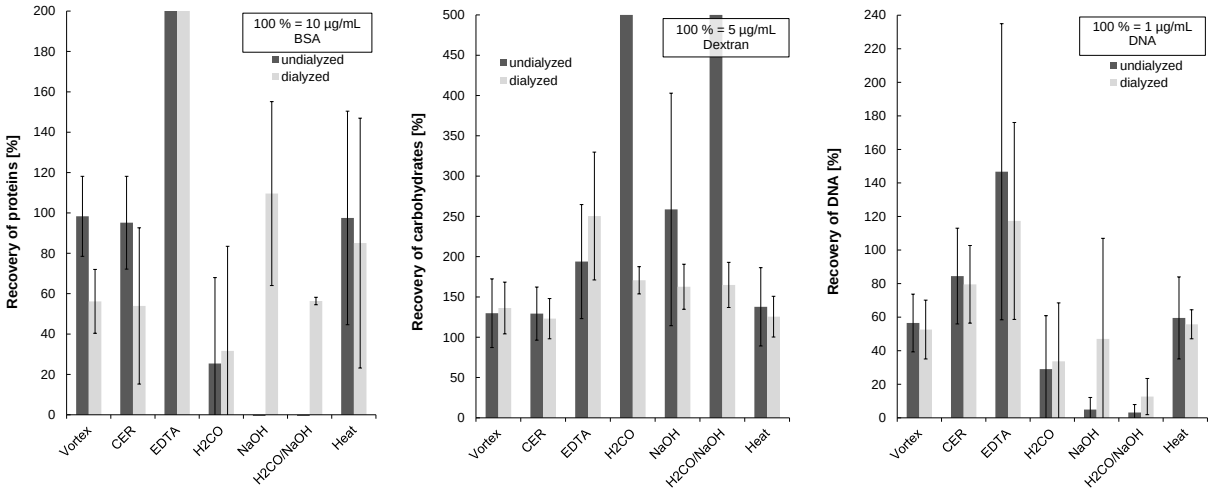

Figure 4.12: Recovery of proteins, carbohydrates and DNA in a model EPS solutions (10 µg mL^{-1} BSA, 5 µg mL^{-1} dextran, 1 µg mL^{-1} DNA) after treatments by shaking (Vortex), CER, formaldehyde, NaOH, formaldehyde/NaOH, EDTA or heat as used for the isolation of EPS from drinking-water biofilms. Proteins were quantified by the modified Lowry assay (Peterson, 1977), carbohydrates were determined by the sulphuric acid/phenol method, DNA was analyzed by PicoGreen (n = 2 independent measurements).

4.3.2 Qualitative comparison of isolated EPS proteins

The isolation procedures were further evaluated for the suitability of the isolated EPS proteins for qualitative analysis by 2D gel electrophoresis. Shaking treatment was used as reference and showed 198 spots in the range between 20 and 200 kDa and a pI of 3 to 10 (Fig. 4.13a). CER treatment resulted in the isolation of a higher diversity of proteins, with up to 650 spots in the same range (Fig. 4.13b). The highest number of protein spots could be found at pH 4 and pH 7 and molecular weights of 21.5 kDa and 55.4 kDa. EPS isolated with formaldehyde/NaOH showed a smear in the acidic region of the gel and only a small number of focused protein spots, including two spots corresponding to the DNase Benzonase used

for sample preparation, which according to the manufacturer consists of 2 subunits, and has a molecular weight of 30 kDa and a pI of 6.85 (Fig. 4.13c). This is most likely due to hydrolysis of the proteins at high pH. EDTA interfered with the focusing step of the 2D gel electrophoresis, therefore, a 2D protein spot pattern could not be produced. Heat isolated EPS showed a higher diversity of proteins (up to 230 spots) compared to shaking, however, the amount of spots was lower compared to CER isolated EPS (Fig. 4.13d).

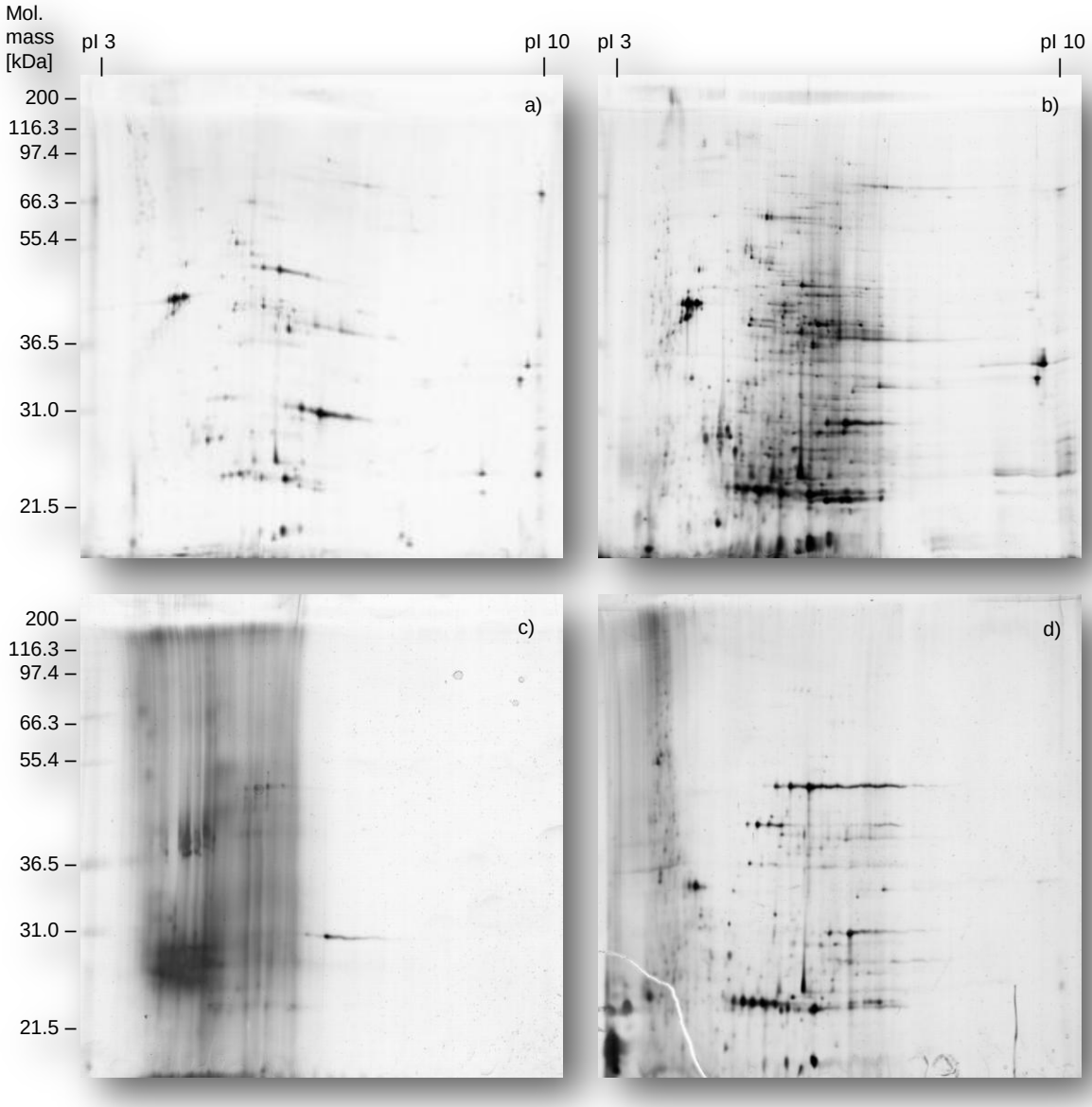

Figure 4.13: 2DE gels of EPS proteins isolated from 14 d-old drinking-water biofilms by a) shaking, b) CER, c) formaldehyde/NaOH or d) heat.

4.3.3 Impact of EPS isolation methods on culturability of biofilm cells

The EPS isolation methods were analyzed for their impact on the culturability of drinking-water biofilm cells after isolation treatment by shaking, CER, formaldehyde/NaOH, EDTA or heat. Recovered cell pellets after the respective isolation treatment were resuspended in same volumes of 6 mM phosphate buffer (pH 7.0), plated on R2A medium and incubated for 7 d at 20 °C.

The control treatment by shaking and the optimized CER treatment showed no significant detrimental effect on the culturability of drinking-water biofilm cells on R2A medium. Similar numbers of colonies were obtained as for the untreated biofilm sample (Fig. 4.14). Treatment with formaldehyde/NaOH, EDTA, or heat resulted in a loss of culturability by > 99.9 %. In the case of formaldehyde/NaOH and heat culturability was below limit of detection (10 cfu).

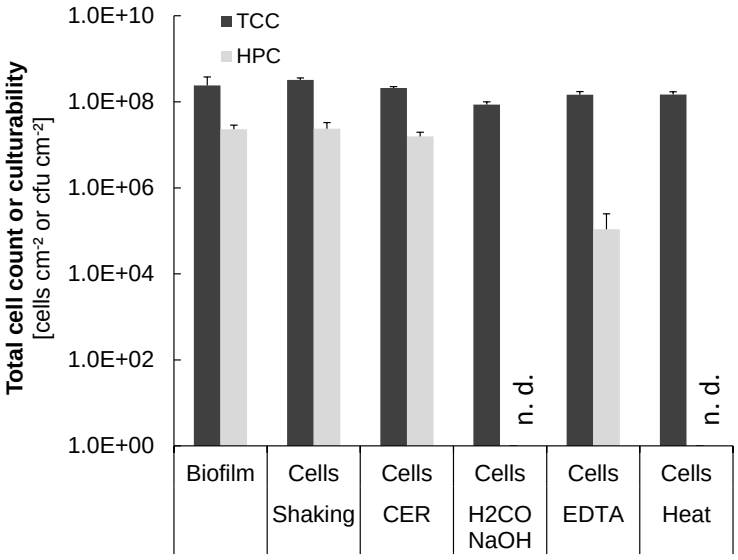

Figure 4.14: Total cell count (TCC) and culturability of drinking-water biofilm cells before and after isolation treatments by shaking, CER, formaldehyde/NaOH, EDTA and heat. Total cell count was determined by DAPI-staining, HPC was analyzed by spread-plate method on R2A medium and enumeration after incubation for 7 d at 20 °C (n = 2 independent reactor runs). n. d., not detected.

4.4 Dynamics of microbial populations and biochemical composition within drinking-water biofilms

Established drinking-water biofilms have been extensively studied in real drinking water distribution systems, and in a low number of studies also in domestic plumbing systems, in terms of total cell count, culturability or population diversity. However, the composition of the EPS matrix of drinking-water biofilms, the dynamics and the function of EPS components throughout the different stages of biofilm formation has not been addressed so far. In this study methods established for the cultivation and analysis of drinking-water biofilms (Sections 4.1 and 4.2) were applied to investigate the colonization of the EPDM substratum by drinking water organisms and the progress of biofilm formation and their EPS with time at two separate locations. Drinking-water biofilms were grown on EPDM coupons for periods of up to 28 d in reactors connected to plumbing system A or plumbing system C (Section 3.1). Coupons were removed twice a week within this period and recovered biofilms and their EPS were analyzed by microbiological, molecular biological or biochemical means. Results represent mean values ± standard deviations of 3 independent reactor runs at plumbing system A or 2 independent reactor runs at plumbing system C.

4.4.1 Progress of surface colonization and drinking-water biofilm formation

Drinking-water biofilms grown at plumbing system A and plumbing system C were analyzed for total cell count with the DAPI method and the proportion of culturable heterotrophic plate count organisms on R2A medium incubated for 7 d at 20 °C.

Cultivation of drinking-water biofilms on EPDM over 28 d resulted in maximum total cell counts of 3.3 x 10^8 cells cm^{-2} (plumbing system A) or 5.1 x 10^8 cells cm^{-2} (plumbing system C) after 21 d of cultivation (Fig. 4.15). In both cases a quasi-stationary state was reached from which on total cell counts remained constant. Biofilms grown at plumbing system A required 14 d of cultivation to reach the quasi-stationary state, with cell counts in the range of 1 x 10^8 cells cm^{-2} and 3.3 x 10^8 cells cm^{-2}, while biofilms grown in plumbing system C showed constant cell counts between 4.1 x 10^8 cells cm^{-2} and 5.1 x 10^8 cells cm^{-2} once the biofilm reached 11 d of age. The HPC of drinking-water biofilm cells was significantly lower

compared to the total cell count, however, the proportion of culturable cells differed depending on the drinking water source and on the biofilm age. Biofilms grown at plumbing system A revealed a relatively high proportion of culturable cells for young biofilms of 5 d to 7 d of age, showing 3.4 x 10^6 cfu cm^{-2} and 3.1 x 10^7 cfu cm^{-2}, which amounted to 43.6 % and 42.8 % of the corresponding total cell count, respectively. The proportion of culturable biofilm cells decreased throughout the experimental run with increasing biofilm age to a minimum of 7.5 % of the total cell count. Biofilms grown at plumbing system C on the other hand, showed relatively constant proportions of culturable cells throughout the entire cultivation period, in the range of 7.4 % to 17.4 % of the corresponding total cell count. The lowest proportion of culturable cells from plumbing system C was detected at the end of the experimental run in 28 d-old biofilms showing a culturability of 7.4 %, which was similar to the culturability of biofilm cells cultivated in drinking water at the plumbing system A.

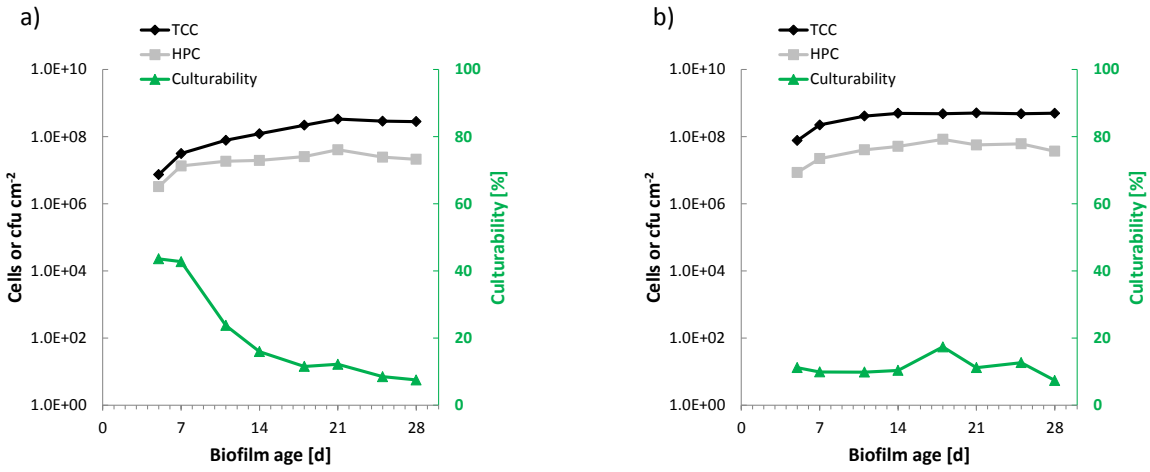

Figure 4.15: Total cell count (TCC) and culturability of drinking-water biofilms grown over periods of up to 28 d in drinking water at a) plumbing system A (n = 3 independent reactor runs) or b) plumbing system C (n = 2 independent reactor runs). Total cell count was determined by DAPI staining, HPC was determined on R2A medium after incubation for 7 d at 20 °C.

4.4.2 Dynamics of microbial populations of drinking-water biofilms over 28 d

The dynamics of microbial populations in drinking-water biofilms grown over periods of up to 28 d at plumbing system A and plumbing system C were analyzed by DGGE of PCR amplified 16S rDNA (PCR-DGGE). DNA was isolated from cell pellets obtained during EPS isolation using the DNeasy Plant Mini Kit (Qiagen) in combination with an implemented bead-beater treatment, and was used as template for PCR.

PCR-DGGE band patterns obtained from drinking-water biofilm cells showed the most significant increase of microbial diversity within the first 5 to 7 days (Fig. 4.16). Biofilms grown at the plumbing system A and aged 3 d revealed a total of 9 bands. Once the biofilms reached 7 d of age band diversity increased to 63 bands and from then on the number and patterns of bands remained relatively uniform. The number of bands in biofilms ≥ 7 d ranged between 60 and 66 well resolved bands with Dice similarity of 70 % to 93 % (Tab. 4.2). Plumbing system C biofilms showed 34 to 45 well resolved bands for biofilms between 5 to 28 d of age. The DGGE band patterns for plumbing system C biofilms exhibited a similarity of 69 % to 99 % throughout the cultivation period. Changes in band intensities were observed. DGGE band patterns of the same samples but from different reactor runs exhibited similarities of 78 % to 93 %.

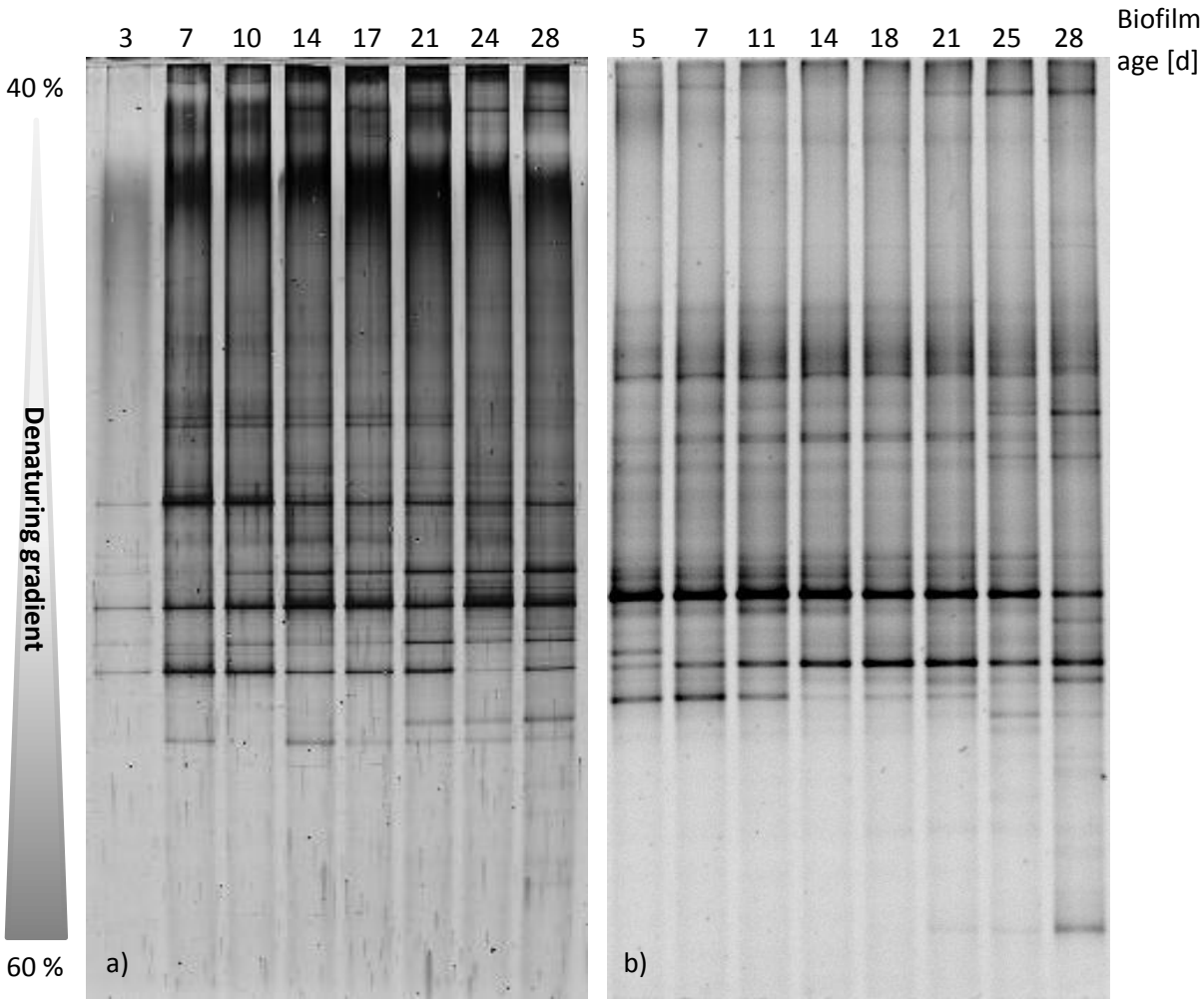

Figure 4.16: PCR-DGGE band patterns of drinking-water biofilms grown over periods of up to 28 d in drinking-water at a) plumbing system A stained with silver (Blum *et al.*, 1987) or b) plumbing system C stained with SybrGold.

Table 4.2: Comparison of the PCR-DGGE band patterns with regards to number of bands and similarity of profiles of drinking-water biofilms grown for up to 28 d at plumbing system A or plumbing system C.

Plumbing system A

Biofilm age [d]	No. of bands	No. of similar bands of biofilms aged								Dice coefficient C_s							
		3 d	7 d	10 d	14 d	17 d	21 d	24 d	28 d	3 d	7 d	10 d	14 d	17 d	21 d	24 d	28 d
3	9	-	8	7	7	8	7	7	8	-	0.22	0.20	0.20	0.22	0.20	0.19	0.21
7	63	8	-	54	45	45	48	46	51	0.22	-	0.88	0.72	0.70	0.78	0.73	0.79
10	60	7	54	-	51	48	47	47	48	0.20	0.88	-	0.84	0.77	0.78	0.76	0.76
14	62	7	45	51	-	59	51	55	51	0.20	0.72	0.84	-	0.93	0.84	0.88	0.80
17	65	8	45	48	59	-	53	56	55	0.22	0.70	0.77	0.93	-	0.85	0.88	0.84
21	60	7	48	47	51	53	-	51	55	0.20	0.78	0.78	0.84	0.85	-	0.83	0.87
24	63	7	46	47	55	56	51	-	57	0.19	0.73	0.76	0.88	0.88	0.83	-	0.88
28	66	8	51	48	51	55	55	57	-	0.21	0.79	0.76	0.80	0.84	0.87	0.88	-

Plumbing system C

Biofilm age [d]	No. of bands	No. of similar bands of biofilms aged								Dice coefficient C_s							
		5 d	7 d	11 d	14 d	18 d	21 d	25 d	28 d	5 d	7 d	11 d	14 d	18 d	21 d	25 d	28 d
5	36	-	35	35	35	28	29	28	28	-	0.99	0.93	0.95	0.80	0.81	0.76	0.69
7	35	35	-	35	35	30	28	28	28	0.99	-	0.95	0.96	0.87	0.79	0.77	0.70
11	39	35	35	-	35	28	30	31	32	0.93	0.95	-	0.91	0.77	0.80	0.81	0.76
14	38	35	35	35	-	33	30	32	32	0.95	0.96	0.91	-	0.92	0.81	0.84	0.77
18	34	28	30	28	33	-	32	32	32	0.80	0.87	0.77	0.92	-	0.91	0.89	0.81
21	36	29	28	30	30	32	-	35	36	0.81	0.79	0.80	0.81	0.91	-	0.95	0.89
25	38	28	28	31	32	32	35	-	37	0.76	0.77	0.81	0.84	0.89	0.95	-	0.89
28	45	28	28	32	32	32	36	37	-	0.69	0.70	0.76	0.77	0.81	0.89	0.89	-

4.4.3 Changes in biochemical composition of drinking-water biofilms over 28 d

Drinking-water biofilms cultivated on EPDM over periods of up to 28 d at plumbing system A and plumbing system C and isolated EPS were biochemically characterized for their protein, carbohydrate and eDNA content by photometric and fluorometric methods. Concentrations were calculated per biofilm surface area or per cell.

Both biofilms exhibited relatively similar concentrations of total and EPS components throughout biofilm cultivation of up to 28 d. Proteins represented the main component of drinking-water biofilms and their EPS irrespective of biofilm age and the type of drinking water, followed by carbohydrates and DNA (Fig. 4.17 and Fig. 4.18). Calculated per surface area, plumbing system C biofilms initially allowed for a higher production of all components compared to plumbing system A biofilms.

- Proteins -

The progress of protein production demonstrated similarities in biofilms grown at both cultivation sites. Calculated per surface area, proteins displayed a gradual increase of total protein to maximum concentrations of 74.5 ± 19.0 µg cm^{-2} after 28 d at plumbing system A

and 81.7 ± 26.1 µg cm^{-2} after 21 d at plumbing system C (Fig. 4.17 and Fig. 4.18, upper diagrams). EPS proteins also showed a gradual increase of concentrations throughout the cultivation period, however, the increase of EPS proteins in biofilms grown at plumbing system A was more pronounced, reaching values of 9.7 ± 2.9 µg cm^{-2} after 28 d, compared to EPS proteins from plumbing system C biofilms, which showed a maximum concentration of 6.2 ± 2.3 µg cm^{-2} after 21 d. The overall progress of biochemical composition of drinking-water biofilms as well as of their EPS did not correlate with total cell counts and calculation of proteins produced per cell exhibited an altered development compared to calculations per surface area (Fig. 4.17 and Fig. 4.18, lower diagrams). Both biofilms exhibited a considerable decrease of protein amounts expressed per cell for the initial stages of biofilm formation of up to 21 d, followed by an increase until the end of the experimental run. EPS protein concentrations of both biofilms also displayed an initial decrease of concentrations for the first 7 d of cultivation. From then on, EPS protein concentrations from plumbing system A biofilms increased continuously to 35.5 ± 12.9 fg $cell^{-1}$ after 28 d. EPS proteins from plumbing system C biofilms, on the other hand, showed only a slight increase in the further progress of biofilm formation to maximum concentrations of 12.0 ± 1.4 fg $cell^{-1}$.

- *Carbohydrates* -

Carbohydrate concentrations of biofilms grown at plumbing system A exhibited a similar trend in progress as was the case for proteins. Considered per surface area, concentrations of total as well as EPS carbohydrate concentrations increased continuously throughout the cultivation period (Fig. 4.17, upper diagrams). Total carbohydrates calculated per cell showed a similar decrease of concentrations from day 5 to day 21, as was the case for proteins, with a subsequent increase until the end of the experimental run (Fig. 4.17, lower diagrams). Carbohydrate concentrations of biofilms grown at plumbing system C showed an altered progress compared to carbohydrates from plumbing system A biofilms. Total carbohydrate concentration calculated per biofilm area showed a saturation curve-like development, showing a strong increase for the initial 14 d of cultivation and from this day on fluctuating concentrations in the range between 18.8 ± 1.7 µg cm^{-2} and 28.3 ± 4.0 µg cm^{-2} (Fig. 4.18, upper diagrams). EPS carbohydrates isolated from plumbing system C biofilms

considered per surface area showed a continuous increase of concentration for the initial 18 d, and from then on relatively constant values. Calculated per cell, total carbohydrate concentration from plumbing system C biofilms decreased for the initial 18 d and from then on remained in the range of 32.1 ± 4.0 fg $cell^{-1}$ and 47.1 ± 6.6 fg $cell^{-1}$ (Fig. 4.18, lower diagrams). EPS carbohydrate concentration produced per cell showed relatively constant values, with highest concentrations at the beginning of the experimental run.

- eDNA -

Extracellular DNA in biofilms grown at plumbing system A as well as at plumbing system C exhibited a saturation curve-like development of concentrations calculated per surface area (Fig. 4.17 and Fig. 4.18, upper diagrams). Plumbing system A biofilms showed the most significant increase in eDNA concentration for the initial 21 d, from which on the concentration increased only slightly to values of 0.36 ± 0.06 µg cm^{-2} after 28d. eDNA concentrations in plumbing system C biofilms increased more steeply, reaching constancy after 14 d at concentrations of 0.47 ± 0.19 µg cm^{-2}. The concentrations of eDNA determined per cell displayed a sharp decline with increasing biofilm age for the initial 18 d (plumbing system A biofilms) or 11 d (plumbing system C biofilms) and in both cases remained constant until the end of the experimental run (Fig. 4.17 and Fig. 4.18, lower diagrams).

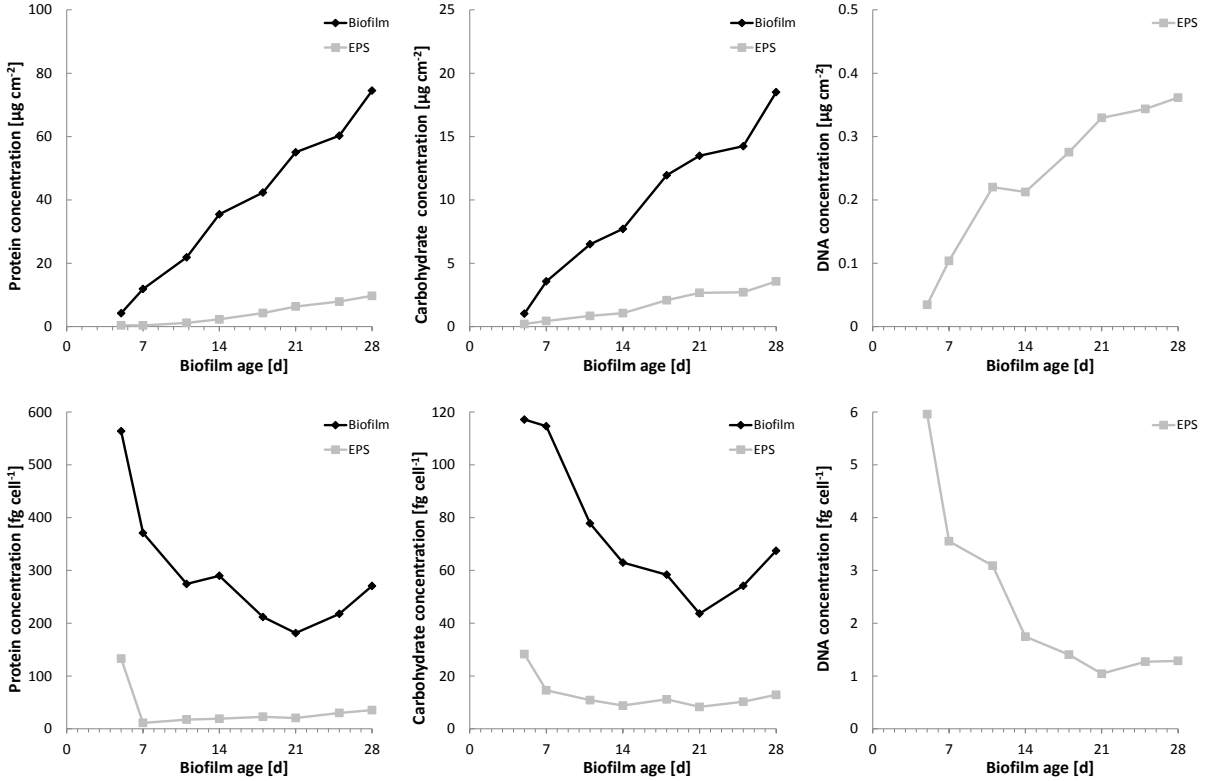

Figure 4.17: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and their EPS grown over periods of up to 28 d in drinking water at plumbing system A. Concentrations were calculated per biofilm surface area (upper diagrams) or per cell (lower diagrams). n = 3 independent reactor runs.

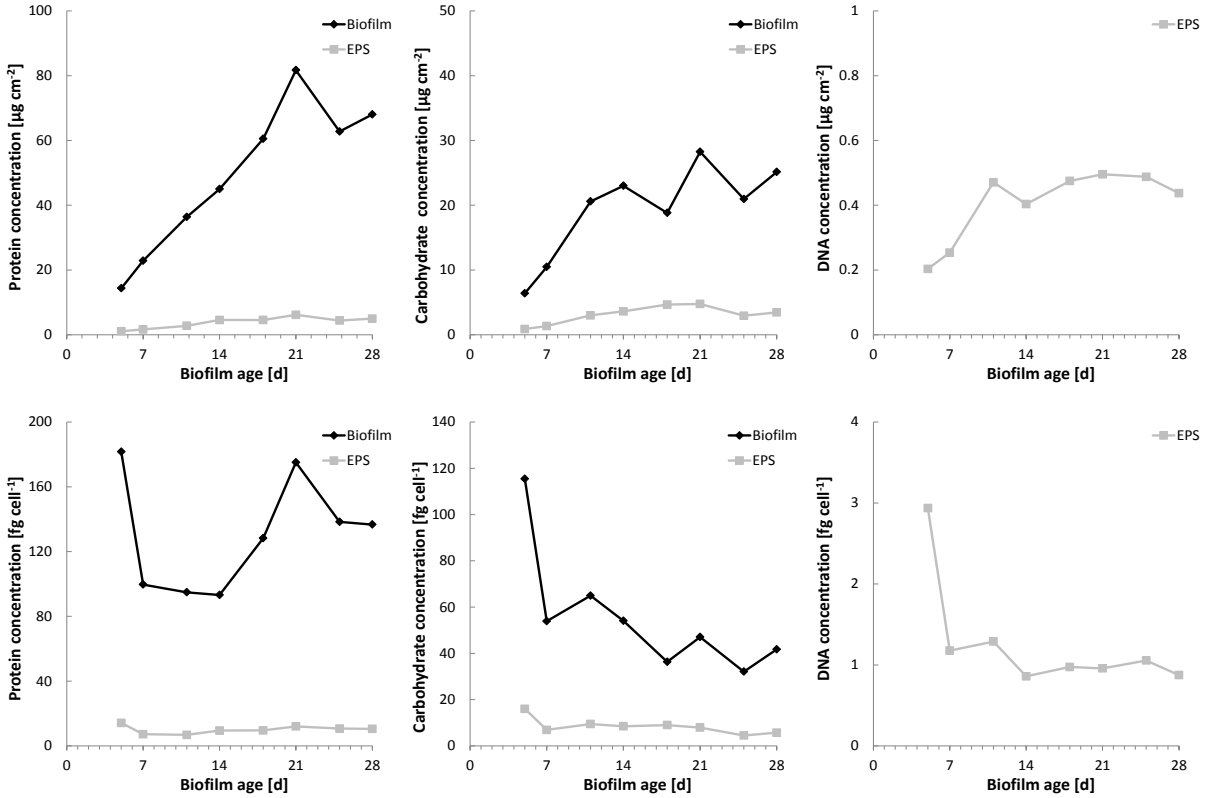

Figure 4.18: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and their EPS grown over periods of up to 28 d in drinking water at plumbing system C. Concentrations were calculated per biofilm area (upper diagrams) or per cell (lower diagrams). n = 2 independent reactor runs.

4.4.4 2DE analysis of the dynamics of EPS proteins from drinking-water biofilms

2DE was applied to generate 2-dimensional spot patterns of EPS proteins at different stages of biofilm formation of drinking-water biofilms. The spot patterns were produced in pH regions between pH 3 and 10 (linear gradient) and molecular weightes of 21.5 kDa and 200 kDa. The 2DE analysis was performed in two separate reactor runs at plumbing system A. The diversity of EPS proteins from drinking-water biofilms cultivated at plumbing system A increased with increasing biofilm age for the first 7 d of cultivation to 520 ± 23 spots and remained relatively constant until day 18 (Tab. 4.3, Fig. 4.19). The majority of spots lay in the range between pH 4 and 8 and molecular weightes of 21.5 kDa and 97.4 kDa. Also a number of acidic proteins (< pI 4) as well as a few basic proteins (> pI 9) could be detected. The number of spots decreased significantly for biofilms > 18 d, showing a total number of 257 ± 89 spots for 28 d-old biofilms at the end of the experimental run. The decrease of spots was most apparent for high molecular weight proteins and for proteins with a high pI. After 18 d of cultivation EPS proteins with molecular weightes > 55.4 kDa as well as EPS proteins with a pI > 7 disappeared completely. Also the two spots which were attributed to the DNase Benzonase (Fig. 4.19, encircled in red) used during sample clean-up disappeared. The decrease of the number, size and pI of proteins and the absence of the Benzonase twin-spots in samples > 14 d of age indicated enzymatic degradation of proteins by proteases. A small number of proteins, however, could be detected throughout all stages of the cultivation (Fig. 4.19, encircled in green), indicating their importance throughout biofilm formation and maintenance.

Table 4.3: Number of proteins in the EPS isolated from drinking-water biofilms grown for up to 28 d in plumbing system A or plumbing system C resolved by 2DE. n = 2 independent reactor runs for plumbing system A biofilms. n = 1 reactor run for plumbing system C biofilms.

Biofilm age	No. of protein spots	
[d]	Plumbing system A	Plumbing system C
5	219 ± 71	573
7	520 ± 23	578
10-11	485 ± 235	446
14	475 ± 240	523
18	509 ± 94	440
21	406 ± 26	479
25	354 ± 88	172
28	257 ± 89	150

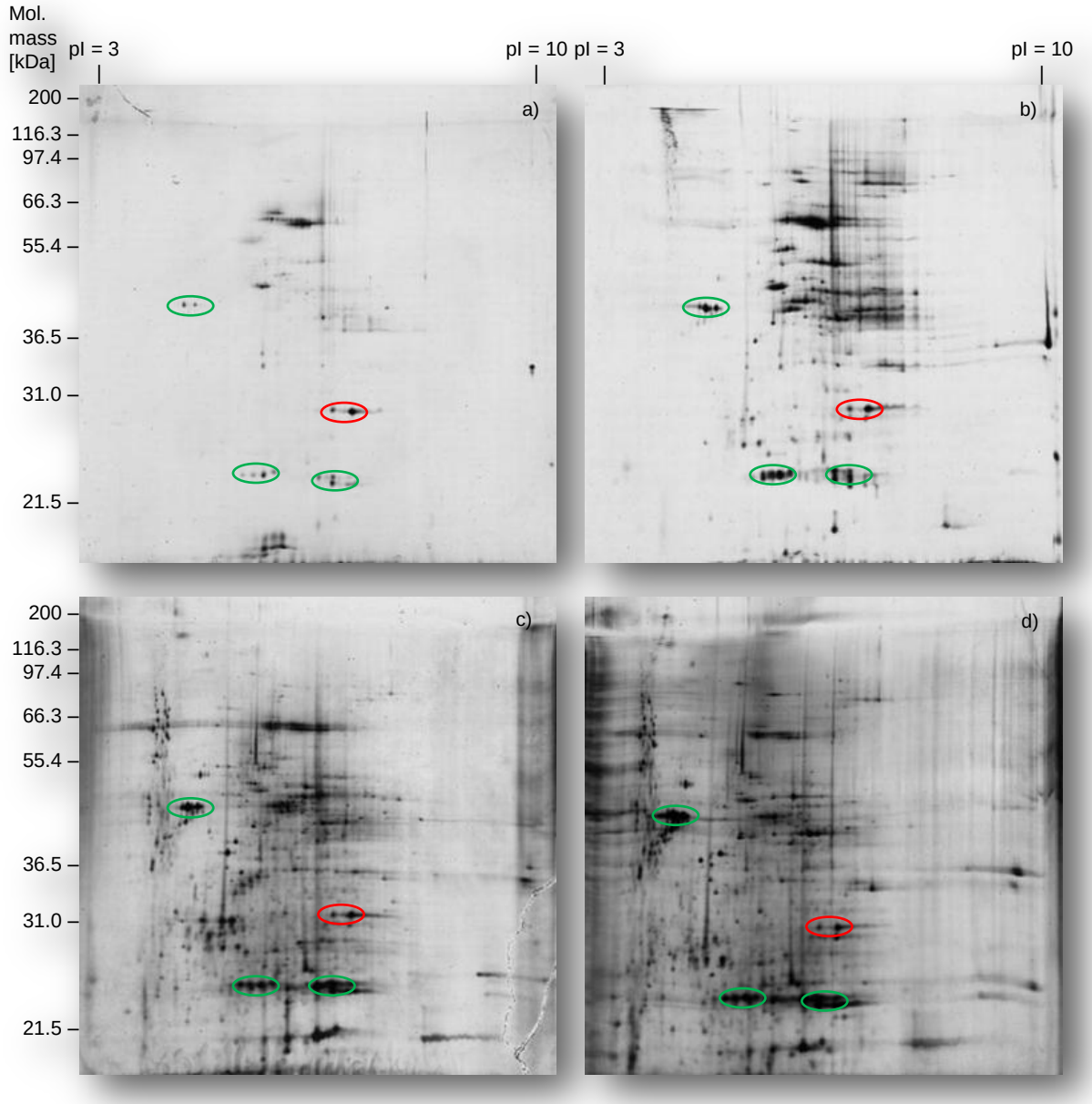

Figure 4.19: 2DE gels of EPS isolated from drinking-water biofilms grown on EPDM at plumbing system A for a) 5 d, b) 7 d, c) 11 d, d) 13 d, e) 18 d, f) 21 d, g)25 d or h) 28 d without the use of a protease inhibitor. Proteins were separated in the first dimension using a linear pH gradient between pH 3 and 10 and a 12 % polyacrylamide gel in the second dimension. Encircled in green: protein spots detected throughout all stages of biofilm formation; encircled in red: protein spots corresponding to the DNase Benzonase. Protein load: 50 µg.

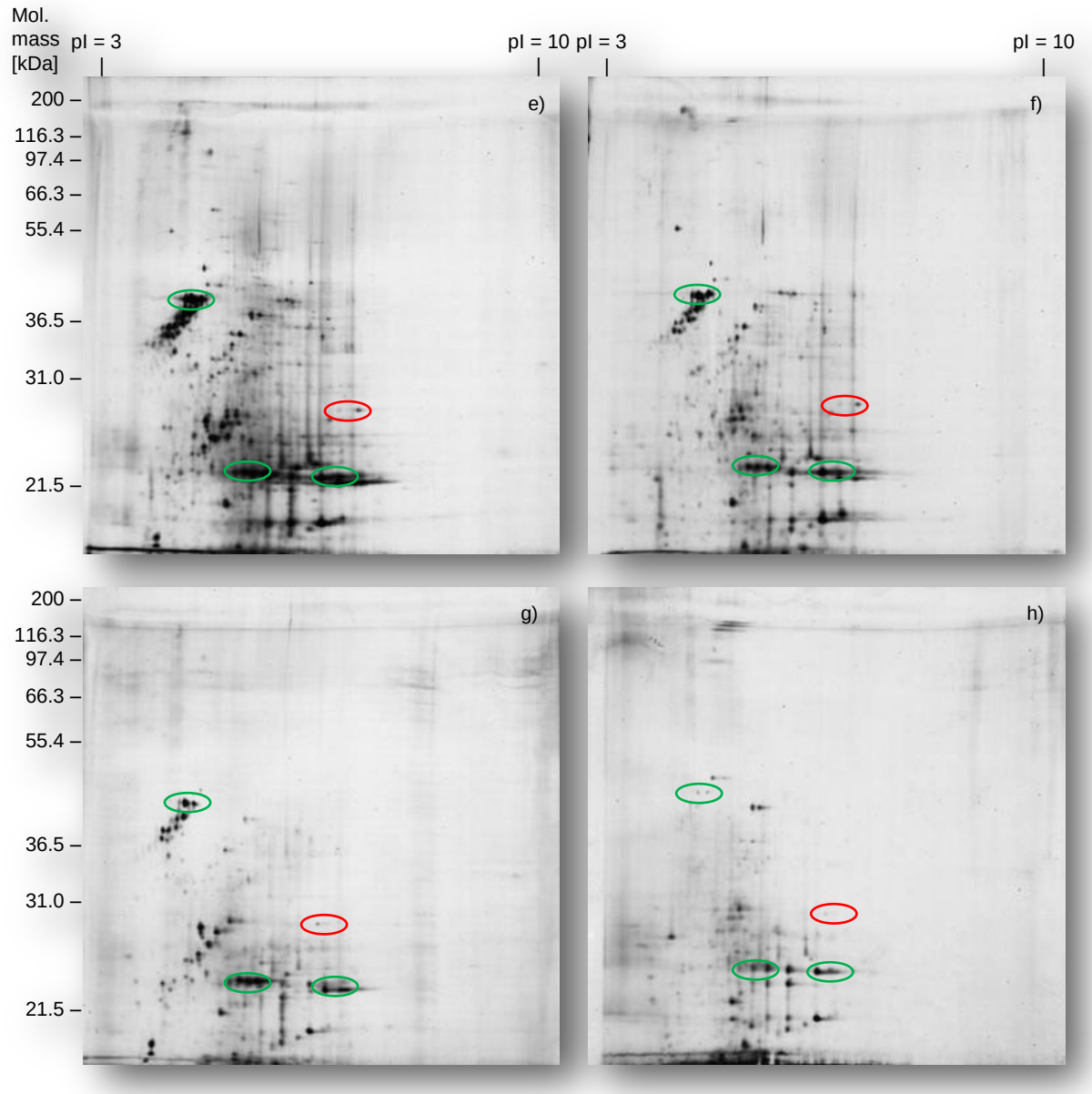

Figure 4.19: *Continued*

Visualization of the extracellular proteins of drinking-water biofilms cultivated at plumbing system C was visualized by 2DE with minor alteration of the procedure applied for EPS proteins of drinking-water biofilms from plumbing system A. A protease inhibitor cocktail (Sigma) was added to the biofilm during sampling, as soon as the biofilm was scraped off the EPDM coupons. Further sample preparation was carried out as previously described (Sections 3.2, 3.8 and 3.12).

The 2DE gels of EPS proteins isolated from drinking-water biofilms cultivated at plumbing system C displayed interference of the protease inhibitor cocktail during the IEF. The application of the protease inhibitor cocktail resulted in prolonged isoelectric focusing and less well resolved protein spots compared to plumbing system A EPS proteins, as well as pronounced horizontal streaking (Fig. 4.20). Enumeration of protein spots was realizable, however, due to the interference, analysis of EPS proteins from plumbing system C biofilms in combination with the protease inhibitor cocktail was performed only once. The highest diversity of proteins was visible after 5 to 7 days of cultivation, showing 573 or 578 spots, respectively. Also in the case of plumbing system C biofilms, the majority of spots was located in the region between pH 4 and 8 and molecular weightes of 21.5 kDa to 97.4 kDa. The number of spots decreased with increasing biofilm age to a minimum of 150 spots after 28 d of cultivation. Similarly to the EPS proteins isolated from drinking-water biofilms from plumbing system A, EPS proteins with large molecular weightes and high pI disappeared completely towards the end of the experimental run.

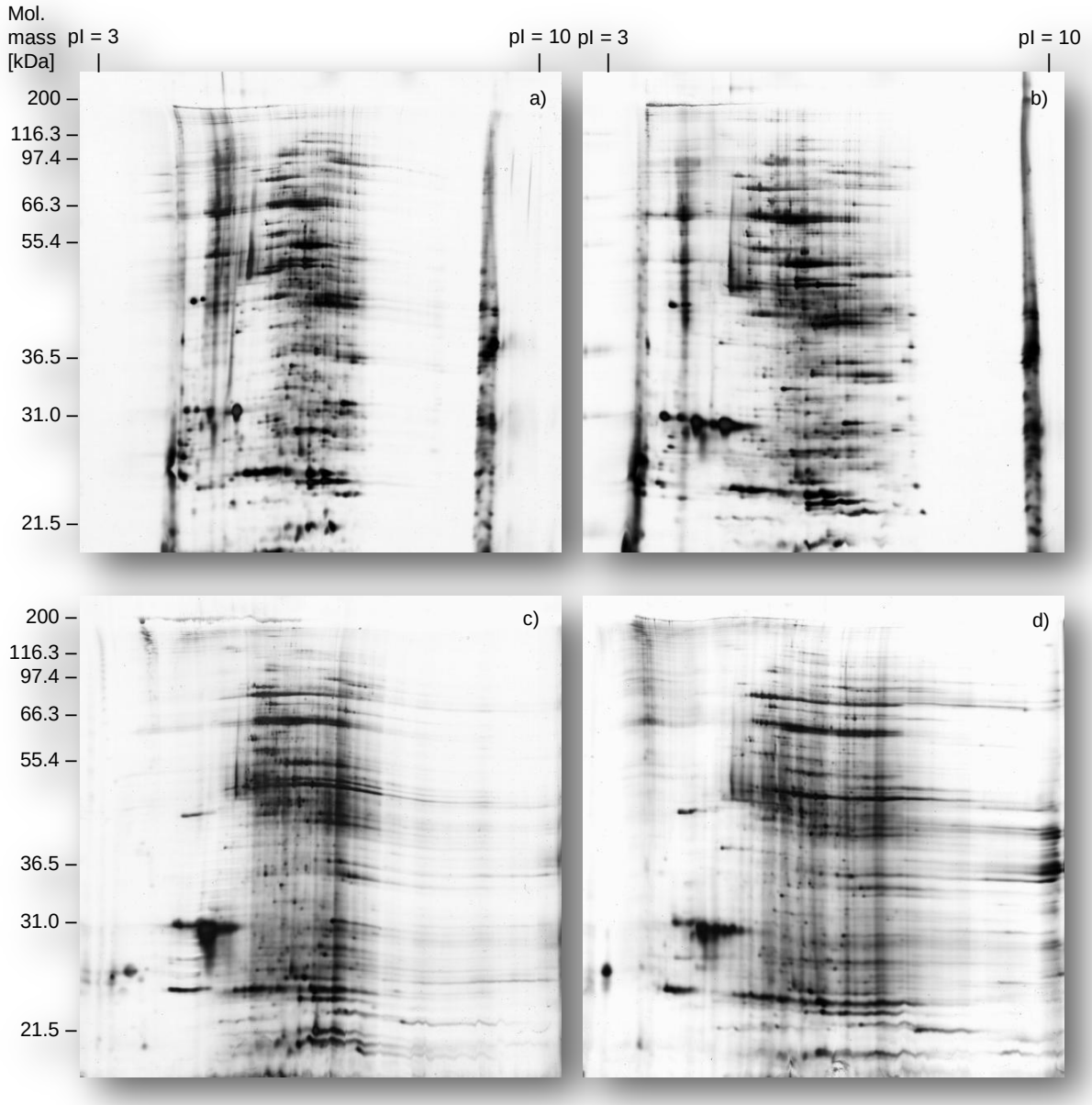

Figure 4.20: 2DE gels of EPS isolated from drinking-water biofilms grown on EPDM at plumbing system C for a) 5 d, b) 7 d, c) 11 d, d) 14 d, e) 18 d, f) 21 d, g)25 d or h) 28 d with the use of a protease inhibitor cocktail. Proteins were separated in the first dimension using a linear pH gradient between pH 3 and 10 and a 12 % polyacrylamide gel in the second dimension. Protein load: 50 µg.

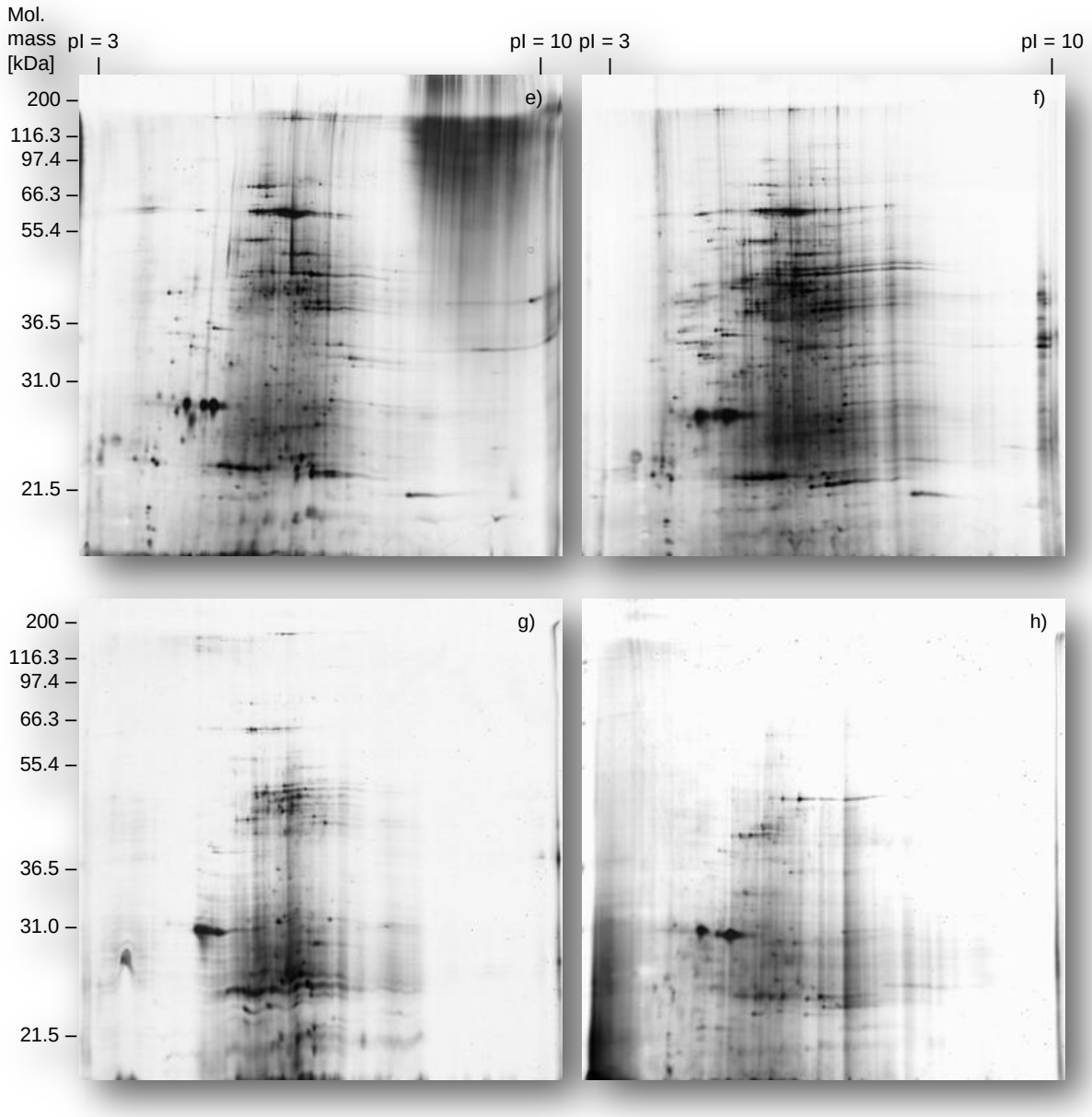

Figure 4.20: *Continued*

4.4.5 Dynamics of proteases in drinking-water biofilms

2DE of EPS proteins isolated from drinking-water biofilms cultivated for up to 28 d in plumbing system A as well as plumbing system C indicated alteration of the protein pattern, showing decreased diversity of EPS proteins with lower molecular weights and pI once the biofilms were > 14 d of age. A hypothesis for this occurrence could be increased production of extracellular proteases, which could cause proteolytic degradation of extracellular proteins. Drinking-water biofilms cultivated at plumbing system C were used to confirm

presence of proteases in drinking-water biofilms and their EPS and their progress during biofilm formation. Protease activity was determined by use of gelatin-containing zymogram gels, which, in presence of protease activity, show clear activity bands at the sites of protease action. Casein-blue zymogram gels proved to be less sensitive compared to gels containing gelatin as substrate.

The determination of proteases with zymogram gels could not detect presence of proteases in 5 d-old or 7 d-old biofilms (Fig. 4.21a) as well as isolated EPS (Fig. 4.21b). Once the biofilms were 11 d of age slightly cleared areas on the gels in molecular weight regions > 62 kDa appeared in the biofilm sample and, less pronounced, in the EPS, however, distinct bands were not obtained. The cleared areas on the gels increased with increasing biofilm age. After 14 d of cultivation 5 faint but resolved activity bands could be visualized in the biofilm sample as well as 3 bands in the EPS sample. The number of bands increased to a maximum of 13 bands in the biofilm sample and 9 bands in the EPS in 28 d-old drinking-water biofilms. Biofilm suspensions or EPS solutions heated at 98 °C for 30 min served as negative controls and did not produce activity bands, irrespective of biofilm age.

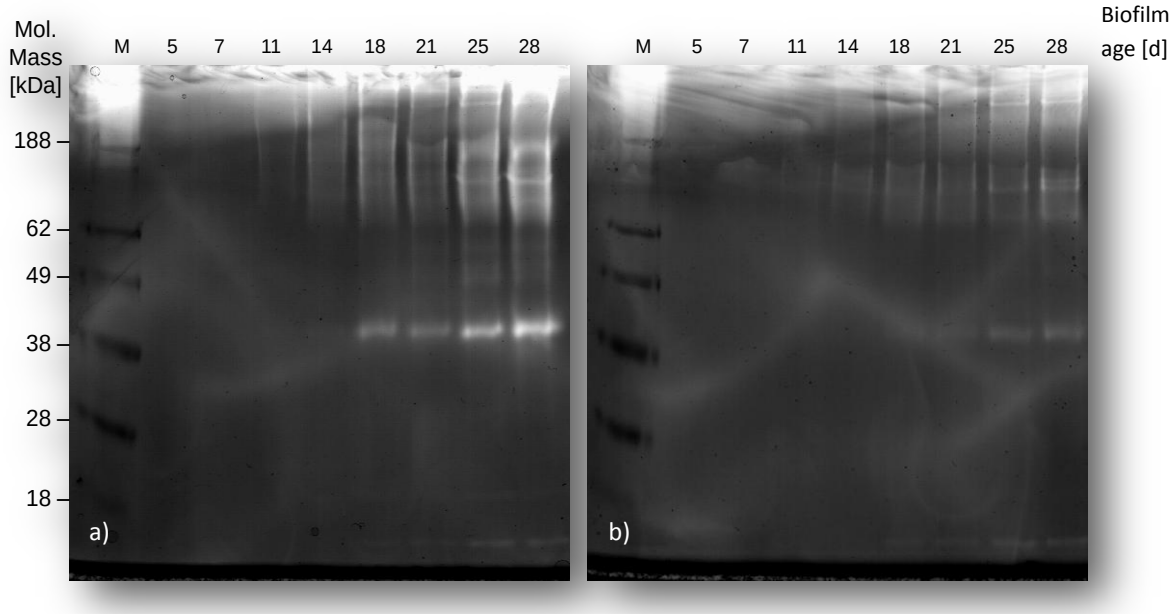

Figure 4.21: One-dimensional zymogram gels showing protease activity in a) drinking-water biofilms suspensions (protein load per lane: 8.5 µg) or b) EPS solutions (protein load per lane: 0.4 µg) isolated from drinking-water biofilms after cultivation for up to 28 d in plumbing system C using zymogram gels containing gelatin as substrate. Gels were stained with the commercially available Simply Blue Safe Stain. Cleared bands indicate degradation of gelatin by proteases.

4.4.6 Enzymatic activity within drinking-water biofilms

Drinking-water biofilms grown for up to 28 d in plumbing system C and their EPS were analyzed for enzyme activities of enzymes belonging to the classes of peptidases, α-D-glucosidases, ß-D-glucosidases, N-acetyl-ß-D-glucosaminidases, lipases, esterases, as well as phosphatases. Activity of these classes of enzymes has been detected in EPS of various environmental as well as pure culture biofilms. The activity was determined by incubation of total biofilms, biofilm cells after EPS isolation, as well as isolated EPS with ß-naphtylamine or methylumbelliferyl substrates, which, when cleaved, produce a fluorescent signal of a specific wavelength, proportional to the amount of cleaved substrate. Results represent the arithmetic means of two independent measurements.

The determination of enzymatic activity in drinking-water biofilms revealed activities of all enzyme classes in the three fractions, total biofilm, biofilm cells after EPS isolation and isolated EPS. Generally, with minor exceptions, total biofilm suspensions exhibited highest absolute enzyme activity, followed by the cell suspension and isolated EPS solution (Fig. 4.22). Esterases demonstrated highest activities throughout the cultivation period, followed by lipases, N-acetyl-ß-D-glucosaminidases, phosphatases, peptidases, α-D-glucosidases and ß-D-glucosidases. Lipases, esterases and phosphatases revealed a gradual increase in enzyme activity during the cultivation period (Fig. 4.22, lower diagrams). Peptidases, α-D-glucosidases, ß-D-glucosidases and N-acetyl-ß-D-glucosaminidases on the other hand exhibited a progress similar to a general cultivation curve, showing a “lag phase” during the first 11 to 18 d of biofilm age, and an “exponential phase” between days 18 and 28 (Fig. 4.22, upper diagrams).

Considering specific activity, total biofilm or cell suspension exhibited a rather constant proportion of hydrolytic enzymes throughout the cultivation run (Fig. 4.23). Specific activity within the EPS on the other hand, in the case of peptidases, α-D-glucosidases ß-D-glucosidases, and N-acetyl-ß-D-glucosaminidases displayed a significant increase of specific enzymatic activity once biofilms reached 14 d of age. Specific activities of lipases, esterases and phosphatases in the EPS demonstrated a gradual increase.

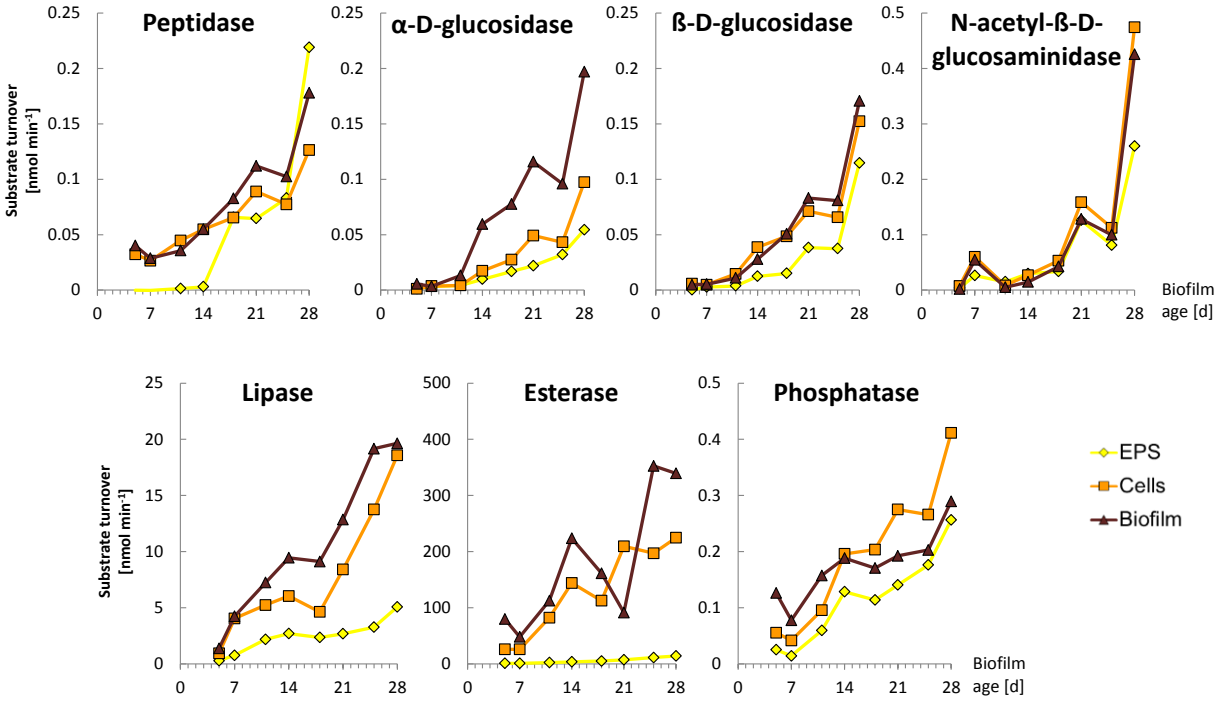

Figure 4.22: Total enzyme activities expressed in nmol min^{-1} of seven enzyme classes determined in the whole biofilm, in biofilm cells after EPS isolation, or in isolated EPS obtained from drinking-water biofilms after cultivation for up to 28 d. n = 2 independent reactor runs.

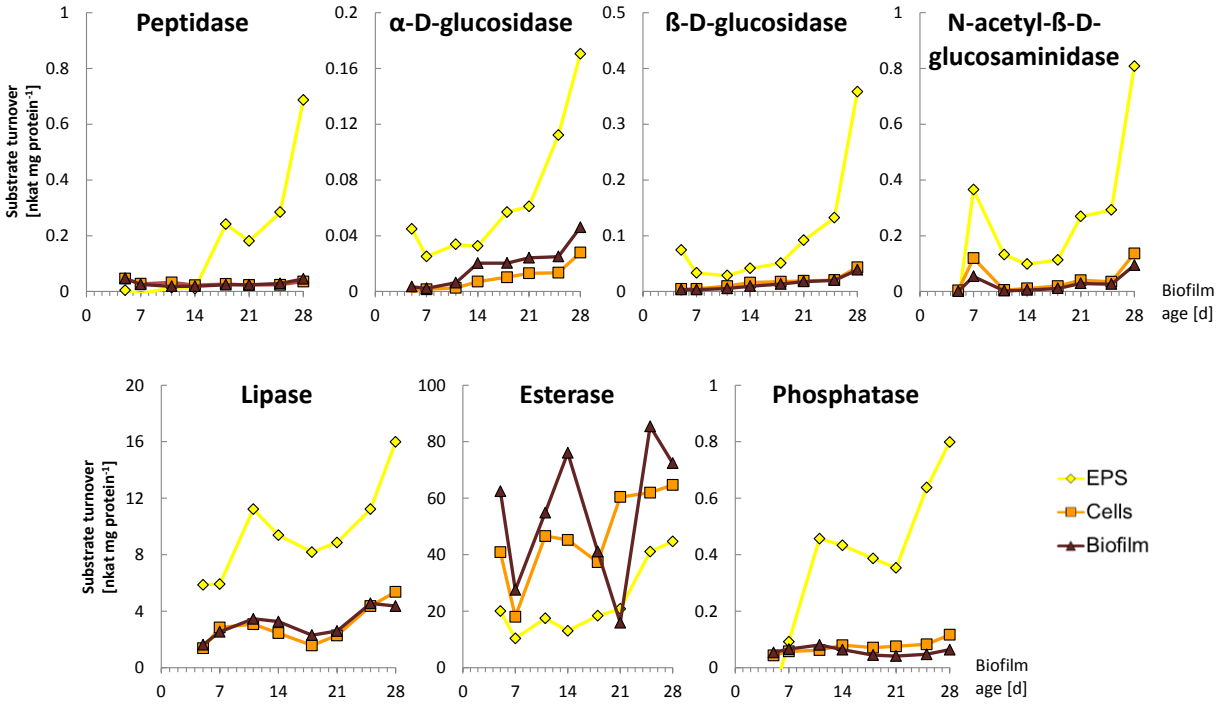

Figure 4.23: Specific enzyme activities expressed in nkat mg^{-1} protein of seven enzyme classes determined in the whole biofilm, in biofilm cells after EPS isolation, or in isolated EPS obtained from drinking-water biofilms after cultivation for up to 28 d. n = 2 independent reactor runs.

4.5 Comparison of drinking-water biofilms grown at different locations

Characteristics of drinking water can have substantial influence on the development of drinking-water biofilms. Temperature and pH are in general major factors determining biofilm growth. Additionally, the chemical composition of bulk water can have a direct impact on microorganisms in the planktonic or sessile state (Fletcher, 1988; Donlan, 2002). These parameters can vary considerably among drinking waters from different distribution systems and in particular once drinking water passes through plumbing systems. In the present study established methods for biofilm cultivation, EPS isolation and analysis were applied to investigate the influence of drinking water quality as well as the influence of plumbing materials made of Cu on biofilm formation, and to determine variability of composition among drinking-water biofilms and their EPS. Drinking-water biofilms were cultivated at five different locations for 14 d on EPDM coupons inserted into flow-through reactors, which were connected to the respective water system. The locations were distribution system A and plumbing system A, both supplied by water supplier A, distribution system B and plumbing system B, both supplied by water supplier B, and plumbing system C, supplied by water supplier C (Section 3.1). 14 d-old drinking-water biofilms from all sites were analyzed for total cell count, HPC, population diversity and biochemical composition of total biofilms and their EPS in three independent reactor runs. Wet weights, dry weights, loss and residue on ignition, as well as water contents were determined in separate biofilm cultivation runs. Temperature and free chlorine concentrations were monitored regularly throughout the cultivation runs. Drinking water samples were collected from all location at the end of two separate cultivation runs from the same taps, which fed the biofilm reactors, and analyzed for their composition of inorganic substances.

4.5.1 Drinking water composition and characteristics at the cultivation sites

Substantial differences in water characteristics between the drinking waters supplied by the three water suppliers A, B and C were detected, in particular regarding water temperature, pH, water hardness, Ca and Mg concentrations and conductivity (Tab. 4.4). During the experimental periods temperature fluctuations were relatively high for drinking waters

obtained at plumbing system A, distribution system B and plumbing system B. The multi-element analysis showed that all inorganic substances were present in the drinking waters at concentrations below the permissible limit values set by the German Drinking Water Ordinance. Differences in composition of inorganic substances were in particular evident for Ca and Mg, which were among the most abundant ions in all drinking waters. Plumbing system C, furthermore, revealed highest Fe concentrations (0.12 ± 0.01 mg L^{-1}), while Fe in all other drinking waters was below the limit of detection (0.01 mg L^{-1}). Within the same water systems significant changes in Cu concentrations were observed. Drinking waters sampled from plumbing systems, which in all cases were made of Cu, revealed significantly higher Cu concentrations in the drinking water compared to the respective distribution systems. A 61 fold higher concentration was detected in the drinking water sampled from plumbing system A (0.067 ± 0.019 mg L^{-1}) compared to the drinking water sampled from the distribution system A (0.0011 ± 0.0004 mg L^{-1}), while Cu in the drinking water sampled from plumbing system B (0.037 ± 0.001 mg L^{-1}) was 22 fold higher compared to the drinking water at the distribution system B (0.0017 ± 0.0001 mg L^{-1}). The drinking water collected from plumbing system C contained highest concentrations of Cu (0.14 ± 0.04 mg L^{-1}). Concentrations of all other inorganic substances in the drinking waters were similar comparing water collected from distribution systems to those from the respective plumbing systems. Free chlorine concentrations were usually below limit of detection (0.01 mg L^{-1}), with occasional readings of 0.01 mg L^{-1}.

Table 4.4: General characteristics of drinking waters at the cultivation sites of drinking-water biofilms. Temperatures and free chlorine concentrations represent values of ≥ 8 measurements over ≥ 3 independent reactor runs. Concentrations or inorganic substances represent mean values ± standard deviations of 2 independent measurements.

Parameter	Drinking-water biofilm cultivation sites				
	Plumbing system A	Distribution system A	Plumbing system B	Distribution system B	Plumbing system C
Experimental period					
	24 Nov. 2009 - 01 Jun. 2010	24 Nov. 2009 - 01 Jun. 2010	29 Apr. 2010 - 22 Jun. 2010	29 Apr. 2010 - 22 Jun. 2010	13 May 2011 - 25 Aug. 2011
Temperature [°C]					
Mean	17.3 ± 2.9	11.4 ± 0.9	19.0 ± 1.9	19.1 ± 2.5	22.0 ± 0.7
Min.	10.8	10.4	14.5	13.3	20.2
Max.	21.4	13.0	20.3	20.9	23.1
Concentrations of inorganic substances [mg L^{-1}]					
Free Chlorine	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Ca	112.5 ± 3.54	112.0 ± 2.8	49.8 ± 0.2	49.9 ± 0.28	37.7 ± 2.8
K	14.8 ± 15.4	3.9 ± 0.07	5.7 ± 0.4	5.65 ± 0.35	3.3 ± 0.0
Na	25.2 ± 0.78	24.6 ± 0.1	59.0 ± 5.4	59.4 ± 5.0	28.1 ± 1.7
Mg	11.5 ± 0.14	11.45 ± 0.2	8.1 ± 0.1	8.15 ± 0.07	6.6 ± 0.6
Fe	<0.01	<0.01	<0.01	<0.01	0.12 ± 0.008
Mn	0.002 ± 0.000	<0.001	<0.01	<0.01	0.008 ± 0.003
Zn	0.013 ± 0.001	<0.01	0.014 ± 0.001	<0.01	0.016 ± 0.003
Cu	0.067 + 0.019	0.0011 ± 0.0004	0.037 ± 0.001	0.0017 ± 0.0001	0.14 ± 0.04
Al	<0.01	<0.01	<0.01	<0.01	<0.01
B	0.03 ± 0.002	0.03 ± 0.002	0.084 ± 0.006	0.085 ± 0.006	0.043 ± 0.003
Si	4.83 ± 0.17	4.88 ± 0.1	2.33 ± 0.21	2.33 ± 0.20	2.5 ± 0.1
SiO_2	10.4 ± 0.35	10.45 ± 0.2	4.97 ± 0.45	4.99 ± 0.42	5.35 ± 0.21
P	0.32 ± 0.02	0.36 ± 0.02	0.081 ± 0.003	0.082 ± 0.006	0.074 ± 0.003
PO_4	0.97 ± 0.06	1.09 ± 0.1	0.25 ± 0.01	0.25 ± 0.02	0.23 ± 0.01
Cd	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002
Cr	<0.001	<0.001	<0.001	<0.001	<0.001
Ni	<0.002	<0.002	0.002	0.002	<0.002
Pb	<0.002	<0.002	<0.002	<0.002	<0.002
Co	<0.001	<0.001	<0.001	<0.001	<0.001
Ba	0.07 ± 0.002	0.07 ± 0.003	0.041 ± 0.000	0.041 ± 0.000	0.028 ± 0.000
Sr	0.36 ± 0.01	0.36 ± 0.01	0.21 ± 0.001	0.21 ± 0.001	0.15 ± 0.01
V	<0.01	<0.01	<0.004	<0.004	<0.004
Mo	<0.005	<0.005	<0.005	<0.005	<0.005
Data obtained from the respective water works					
		Supplier A	**Supplier B**	**Supplier C**	
pH		7.19	7.75	7.83	
Hardness [°dH]		19.4	8.7	7.8	
Conductivity [µS cm^{-1}]		745	591	507	
TOC [mg L^{-1}]		0.66	0.74	1.1	

4.5.2 General composition of drinking-water biofilms from different locations

Drinking-water biofilms cultivated at the five locations for 14 d on EPDM in all cases exhibited macroscopically visible slime formation on the coupons. On a wet weight basis, cultivation of biofilms in plumbing systems resulted in a more extensive biomass formation compared to biofilms grown in distribution systems (Tab. 4.5). Total biomass wet weight was 2.9 fold higher in biofilms grown at plumbing system A compared to biofilms grown at the distribution system A, while the wet weight of biofilms grown at plumbing system B was 2.1 fold higher compared to biofilms from distribution system B. Biofilms grown at plumbing system A and at the distribution system A showed similar values in respect to dry weight, while plumbing system B biofilms displayed 2.5 fold higher dry weight compared to distribution system B biofilms.

Table 4.5: Physical characteristics of 14 d-old drinking-water biofilms cultivated at five different sites; n ≥ 2.

Parameter	Drinking-water biofilm cultivation sites				
	Plumbing system A	Distribution system A	Plumbing system B	Distribution system B	Plumbing system C
Wet weight [mg cm^{-2}]	11.3 ± 3.6	3.5 ± 1.9	11.1*	5.4*	16.2 ± 7.3
Dry weight [mg cm^{-2}]	0.085 ± 0.027	0.095 ± 0.032	0.18*	0.073*	0.136 ±0.004
Residue on combustion [mg cm^{-2}]	0.010 ± 0.002	0.011 ± 0.003	0.024*	0.012*	0.026 ± 0.004
Loss on combustion [mg cm^{-2}]	0.075 ± 0.026	0.083 ± 0.029	0.16*	0.061*	0.111 ± 0.000
Water content [% w/w]	99.2	96.5	98.3*	98.6*	99.1

*Values represent a single measurement.

The drinking-water biofilms were analyzed for their content of the metal ions Ca, Mg, Fe, Zn, Cu and Al. Ca was the most abundant metal ion in all drinking-water biofilms, showing concentrations in the range of 118.2 ± 31.8 µg g^{-1} wet weight to 250.9 ± 139.2 µg g^{-1} wet weight (Tab. 4.6). Mg was among the most abundant ions in the biofilms, however, concentrations of Mg in biofilms cultivated at distribution system A or plumbing system B could not be detected, potentially due to the dilution of the biofilm samples during sample preparation for ICP-OES measurements. Comparison of the water phase to the biofilm on a

wet weight basis, assuming a wet weight of 1 mL water to be 1 g, revealed an accumulation of the alkaline earth metal ions Ca and Mg by factors of 1.4 to 9.5 within the biofilm. All biofilms exhibited significant enrichment of the heavy metals Fe, Zn, Cu, and Al within the biofilm matrix by 2 to more than 3 orders of magnitude. Fe, Zn, or Al concentrations in the drinking waters were in many cases close to or below limit of detection (0.01 mg L^{-1}), hence, the limit of detection value was used as basis for the calculation of the enrichment factors of these ions. Cu concentrations were in the range of 0.0011 mg L^{-1} to 0.0017 mg L^{-1} in drinking waters collected from the distribution systems and 0.037 mg L^{-1} to 0.14 mg L^{-1} in drinking waters from plumbing systems made of copper, respectively. Biofilms exposed to drinking waters with elevated Cu concentrations (plumbing system A, B or C) showed 10 to 25 higher Cu contents within the biofilms compared to biofilms cultivated at the distribution systems.

Table 4.6: Inorganic composition of 14 d-old drinking-water biofilms cultivated at five different locations. Numbers in brackets represent enrichment factors of the respective cations in the biofilm compared on a wet weight basis compared to the water phase; n.d., not detected. (n ≥ 2 independent reactor runs).

Metal	Concentrations at drinking-water biofilm cultivation sites				
	Plumbing system A [µg g^{-1} wet weight]	**Distribution system A** [µg g^{-1} wet weight]	**Plumbing system B** [µg g^{-1} wet weight]	**Distribution system B** [µg g^{-1} wet weight]	**Plumbing system C** [µg g^{-1} wet weight]
Ca	160.8 ± 88.1 (1.4x)	207.9 ± 70.2 (1.9x)	189.0 ± 86.3 (3.8x)	250.9 ± 139.2 (5.0x)	118.3 ± 31.8 (3.1x)
Mg	33.0 ± 10.8 (2.9x)	n.d.	n.d.	77.1 ± 19.3 (9.5x)	58.0 ± 10.0 (8.8x)
Fe	7.72 ± 2.2 (>772x)	32.7 ± 16.8 (>3266x)	10.9 ± 0.2 (>1094x)	14.1 ± 1.4 (>1414x)	27.2 ± 7.7 (220x)
Zn	0.88 ± 0.16 (70.2x)	2.1 (>210x)	13.2 ± 16.1 (943x)	16.5 ± 20.9 (1272x)	4.04 ± 0.74 (252x)
Cu	36.9 ± 17.6 (548x)	3.54 ± 3.05 (3370x)	96.2 ± 80.7 (2625x)	3.66 ± 2.79 (196x)	91.7 ± 37.1 (668x)
Al	4.28 ± 3.46 (>428x)	7.76 ± 5.04 (>776x)	4.41 ± 0.7 (>441x)	6.01 ± 0.19 (>601x)	3.28 ± 0.40 (>328x)

4.5.3 Microbiological and population analysis of drinking-water biofilms with different origin

The extent of growth of drinking-water biofilms at the five different locations was evaluated on the basis of to their total cell number and the proportion of culturable HPC organisms.

Total cell counts showed relatively similar values between 6.0 x 10^7 cells cm^{-2} to 2.8 x 10^8 cells cm^{-2} in all drinking-water biofilms (Fig. 4.24) which are in accordance to the biofilm dry weight determinations. The culturability of cells varied slightly depending on location of biofilm cultivation. Generally, biofilms cultivated in drinking waters from plumbing systems made of copper revealed lower proportions of culturable cells compared to biofilms grown in drinking waters in distribution systems. Drinking-water biofilm cells from plumbing system A and B biofilms displayed a culturability of 11.4 % and 15.3 % of their total cell count, respectively, while biofilm cells from the distribution system A and B were culturable to 50.5 % and 46.8 %, respectively. Plumbing system C biofilms showed a similar trend as plumbing system A and B biofilms with 8.5 % of culturable cells.

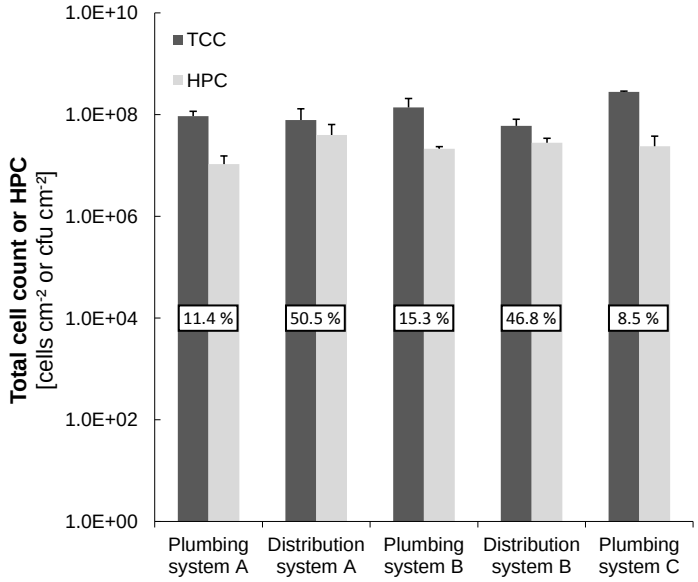

Figure 4.24: Total cell counts (TCC) and HPC of 14 d-old drinking-water biofilms grown at five different locations. Percentage in squares represents the proportion of culturable cells of the respective total cell count (n = 3 independent reactor runs).

Analysis of the population diversity of drinking-water biofilms grown at the five locations was carried out by PCR-DGGE of 16S rDNA fragments of isolated DNA. Similarity of band patterns was expressed by means of the Dice similarity coefficient, on the basis of total number of bands obtained for a biofilm and presence of bands at the same positions in two biofilms.

The comparison of the population diversity of 14 d-old drinking-water biofilms indicated significant differences in DGGE band patterns (Fig. 4.25). Generally, biofilms grown in drinking waters from plumbing systems made of copper exhibited a higher diversity of microorganisms. Drinking-water biofilm cultivated at plumbing system A showed highest diversity of microorganisms, showing 52 bands, followed by the biofilms grown at plumbing system B (46 bands), plumbing system C (43 bands), distribution system A (29 bands), or distribution system B (26 bands). Similarity of band patterns was limited and ranged between 46 % and 63 % (Tab. 4.7). Only three bands were detected at the same positions in the gels for all five drinking-water biofilms (Fig. 4.25, indicated by arrows). DGGE band patterns of the same samples but from different reactor runs exhibited similarities of 65 % to 89 %.

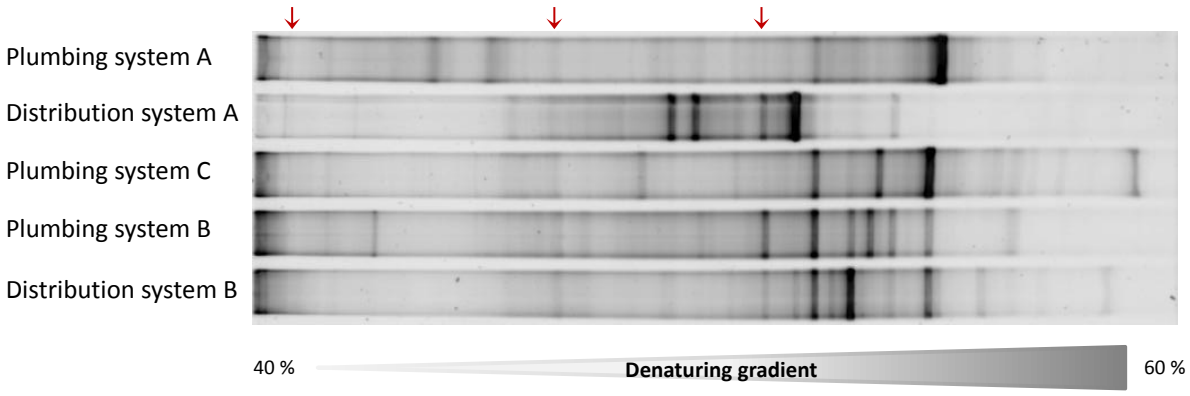

Figure 4.25: PCR-DGGE gel of 14 d-old drinking-water biofilm communities grown at five different locations. Arrows indicate bands at the same positions in all biofilm samples. DNA-load per lane: 750 ng. Staining: SybrGold.

Table 4.7: Number of bands, number of similar bands and similarity of DGGE band patterns of 14 d-old drinking-water biofilm communities grown at five different locations and analyzed by PCR-DGGE.

Biofilm cultivation site	Total No. of bands	No. of similar bands					Dice coefficient C_s				
		Plumbing system A	Distribution system A	Plumbing system C	Plumbing system B	Distribution system B	Plumbing system A	Distribution system A	Plumbing system C	Plumbing system B	Distribution system B
Plumbing system A	52	-	19	30	28	18	-	0.47	0.63	0.57	0.46
Distribution system A	29	19	-	18	20	16	0.47	-	0.50	0.53	0.58
Plumbing system C	43	30	18	-	22	16	0.63	0.50	-	0.49	0.46
Plumbing system B	46	28	20	22	-	20	0.57	0.53	0.49	-	0.56
Distribution system B	26	18	16	16	20	-	0.46	0.58	0.46	0.56	-

4.5.4 Biochemical composition of drinking-water biofilms from different locations

Drinking-water biofilms grown at five different locations as well as their EPS were biochemically analyzed for their protein, carbohydrate and DNA content by photometric or fluorometric assays. Analyzed were total biofilm suspensions, isolated EPS and isolated EPS after dialysis (MWCO 3500 Da). All substances were detected in quantifiable amounts in the biofilms and within their EPS matrix. The overall quantities of polymers as well as the proportion of isolated EPS varied between biofilms with different origin. In all drinking-water biofilms proteins constituted the major component of the biofilm as well as within the EPS matrix, followed by carbohydrates and DNA.

- *Proteins* -

Drinking-water biofilms exhibited variations of protein contents in total biofilms as well as their EPS. Total protein concentrations in the biofilms were in the range between 22.0 ± 1.9 µg cm^{-2} and 41.0 ± 17.6 µg cm^{-2} (Fig. 4.26, upper diagrams). EPS protein concentrations were in the range of 1.4 ± 0.6 µg cm^{-2} and 7.6 ± 1.0 µg cm^{-2}. Biofilms cultivated at plumbing systems made of copper resulted in lower proportions of isolable EPS proteins compared to biofilms cultivated at distribution systems.

Differences in protein contents between biofilms from plumbing systems compared to biofilms from distribution systems become apparent when comparing protein concentrations in the biofilms on a biofilm wet weight basis (Fig. 4.26, lower diagrams). Drinking-water biofilms cultivated at the distribution system A displayed a 2.3 fold higher total protein content compared to the plumbing system A biofilm, while the total protein content of distribution system B biofilms was 1.6 fold higher than the amount found in the plumbing system B biofilm. The EPS protein determination showed 8.8 fold higher content in the EPS of distribution system A biofilms compared to plumbing system A biofilms, and EPS protein concentration in the distribution system B biofilms were 1.2 fold higher than EPS protein amounts in the plumbing system B. Plumbing system C biofilms displayed similar concentrations of total proteins and EPS proteins as plumbing system A biofilms.

EPS proteins were determined in undialyzed as well as in dialyzed (MWCO 3500 Da) EPS samples. Slightly lower protein concentrations were determined in dialyzed samples. The

differences, however, were statistically insignificant ($p > 0.05$), indicating that the major proportion of proteins in the EPS consisted of high molecular weight proteins.

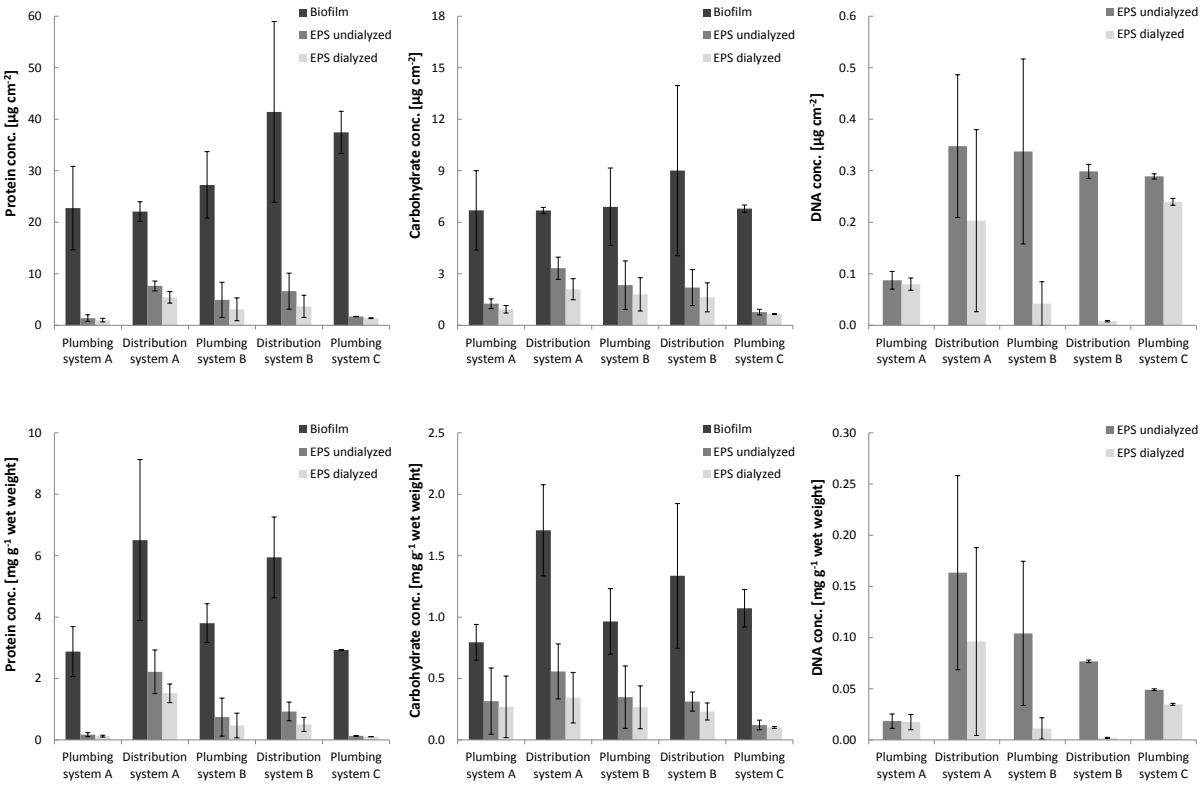

Figure 4.26: Protein-, carbohydrate- and eDNA concentrations in 14 d-old drinking-water biofilms and their EPS. Upper graphs represent concentrations calculated per surface area, lower graphs show concentrations per g wet weight; n = 3 independent reactor runs.

- *Carbohydrates* –

All drinking-water biofilms contained similar total carbohydrate concentrations in the range of 6.7 ± 0.2 µg cm^{-2} and 9.0 ± 5.0 µg cm^{-2} (Fig. 4.26, upper diagrams). Carbohydrates in the EPS showed significant variations in quantity in the range of 0.8 ± 0.2 µg cm^{-2} and 3.3 ± 0.6 µg cm^{-2}.

Similarly to the protein concentrations, also the carbohydrate determination showed a difference of total carbohydrates, when comparing the total carbohydrate concentrations within biofilms from distribution systems to biofilms from plumbing systems on a biofilm wet weight basis (Fig. 4.26, lower diagrams). Distribution system A biofilms displayed a 2.1 times higher carbohydrate content compared to plumbing system A biofilms, while carbohydrate concentrations in distribution system B biofilm were 1.3 times higher compared to plumbing

system B biofilms. Differences considering carbohydrate concentrations in the EPS were less pronounced. The carbohydrate concentration in the EPS of distribution system A biofilms was 1.8 times higher compared to plumbing system A biofilms, and no significant difference was seen for EPS of distribution system B biofilms and EPS of plumbing system B biofilms. EPS of drinking-water biofilms cultivated at plumbing system C showed similar total carbohydrate concentrations and lowest EPS carbohydrate concentrations compared to the 4 other biofilms.

Carbohydrates were determined in undialyzed as well as in dialyzed (MWCO 3500 Da) EPS solutions. Slightly lower carbohydrate concentrations were found in dialyzed samples. The differences were statistically insignificant ($p > 0.05$), indicating the major proportion of determined carbohydrates in the undialyzed sample was high molecular weight polysaccharides.

The proportions of EPS components, which could be isolated by CER, varied among the biofilms. A decreasing trend of EPS yields with increasing Cu concentrations in the drinking waters was observed (Fig. 4.27).

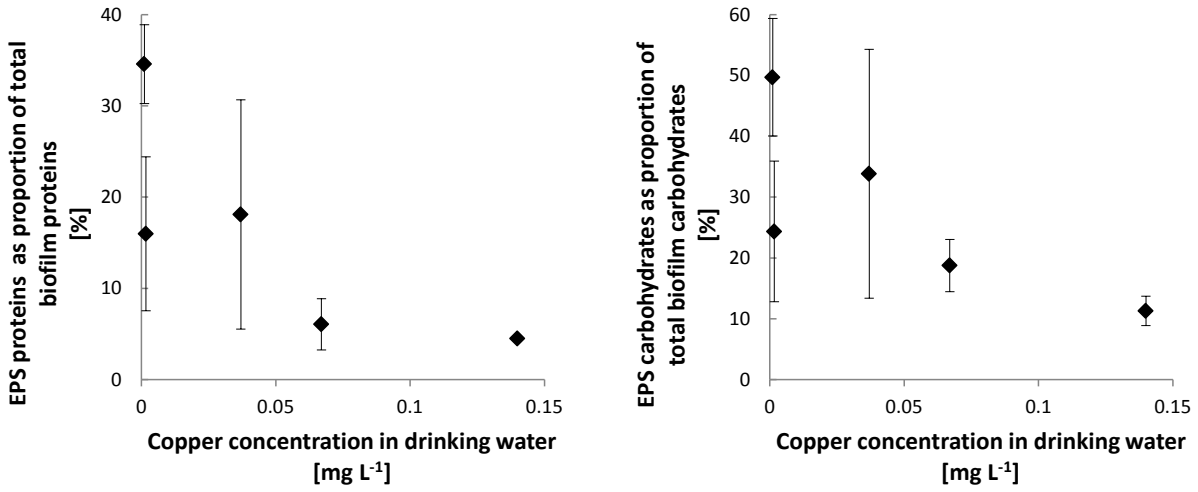

Figure 4.27: Proportions of isolated EPS proteins (left) and carbohydrates (right) from five different drinking-water biofilms in relation to the Cu concentration in the respective drinking water. 100 % represent total drinking-water biofilm proteins or carbohydrates.

- eDNA -

DNA was only determined in undialyzed and dialyzed EPS solutions and showed significant differences in concentration comparing biofilms from the five different locations. Plumbing system A biofilms contained lowest concentrations of eDNA (0.09 ± 0.02 µg cm^{-2}), followed by plumbing system C biofilms (0.29 ± 0.005 µg cm^{-2}), distribution system B biofilms (0.30 ± 0.01 µg cm^{-2}), plumbing system B biofilms (0.34 ± 0.18 µg cm^{-2}) and distribution system A biofilms (0.35 ± 0.14 µg cm^{-2}) (Fig. 4.26, upper diagrams). On a biofilm wet weight basis, eDNA concentrations in distribution system A biofilms were 8.9 times higher compared to plumbing system A biofilms, while eDNA concentrations in distribution system B biofilms were 0.74 times the amount of plumbing system B biofilms (Fig. 4.26, lower diagrams).

Dialysis of EPS solutions in some cases resulted in considerable reductions of eDNA concentrations. While EPS solutions from drinking-water biofilms cultivated at plumbing system A and plumbing system C revealed only 8 % and 17 % lower DNA contents after dialysis, respectively, the eDNA content of distribution system A biofilms, plumbing system B biofilms and distribution system B biofilms was significantly reduced by dialysis by 42 %, 88 % and 97 %, respectively.

- 2DE -

Extracellular proteins were analyzed by 2DE and protein spot patterns were compared for similarity and number of spots. The analysis revealed distinct spot patterns of EPS proteins isolated from 14 d-old drinking-water biofilms cultivated at the five locations (Fig. 4.28). Total numbers of spots varied considerably (Tab. 4.8). In all cases, the majority of spots was located in the pI range between pI 4 and pI 7 and molecular weights of 21.5 kDa and 66.3 kDa. All EPS samples revealed a small number of relatively large proteins with molecular weightes close to 200 kDa, acidic proteins with pI close to pI 3, or, with exception of distribution system B biofilms, also basic proteins with pI close to pI 10 could be visualized (Fig. 4.28).

Similarity of EPS protein spot patterns obtained from the biofilm samples was evaluated based on presence or absence of spots. The similarity of EPS protein spot patterns in general was low. The EPS protein spot pattern of the distribution system B biofilm sample showed

substantial differences to all other spot patterns produced (Fig. 4.28d). In addition to showing the lowest number of spots, only 5 spots with molecular weights > 55.4 kDa and no spots with pI ≥ 8 could be detected. Furthermore, presence of the twin spots corresponding to the DNase Benzonase (Fig. 4.28d; marked in green) was not detected, while their presence was confirmed on 2DE gels of all other EPS samples.

Table 4.8: Number of protein spots detected by 2DE in the EPS of 14 d-old drinking-water biofilms cultivated at 5 different locations in 3 independent reactor runs.

Site of biofilm cultivation	Number of protein spots		
	Run 1	Run 2	Run 3
Plumbing system A	620	617	539
Distribution system A	575	511	761
Plumbing system B	530	436	-
Distribution system B	360	329	-
Plumbing system C	593	425	450

Extracellular protein spot patterns obtained for plumbing system A, distribution system A, plumbing system B and plumbing system C biofilms exhibited some common features. Four protein clusters (Fig. 4.28, encircled in red) were detected in the EPS of the four drinking-water biofilm samples and appeared to be universally present in the EPS of 14 d-old drinking-water biofilms. Three protein clusters (Fig. 4.28, encircled in orange) were found to be only present in the EPS of copper-rich drinking-water biofilms as was the case for biofilms grown at plumbing system A, plumbing system B or plumbing system C. These protein clusters were previously detected in the biofilm dynamics studies (Section 4.4.4) and exhibited prevalence throughout the cultivation of up to 28 d. A total of 36 protein spots, which included spots in common in the EPS of all drinking-water biofilms, or found only in the EPS of plumbing system biofilms were selected for identification by MALDI-TOF-MS (Section 4.6).

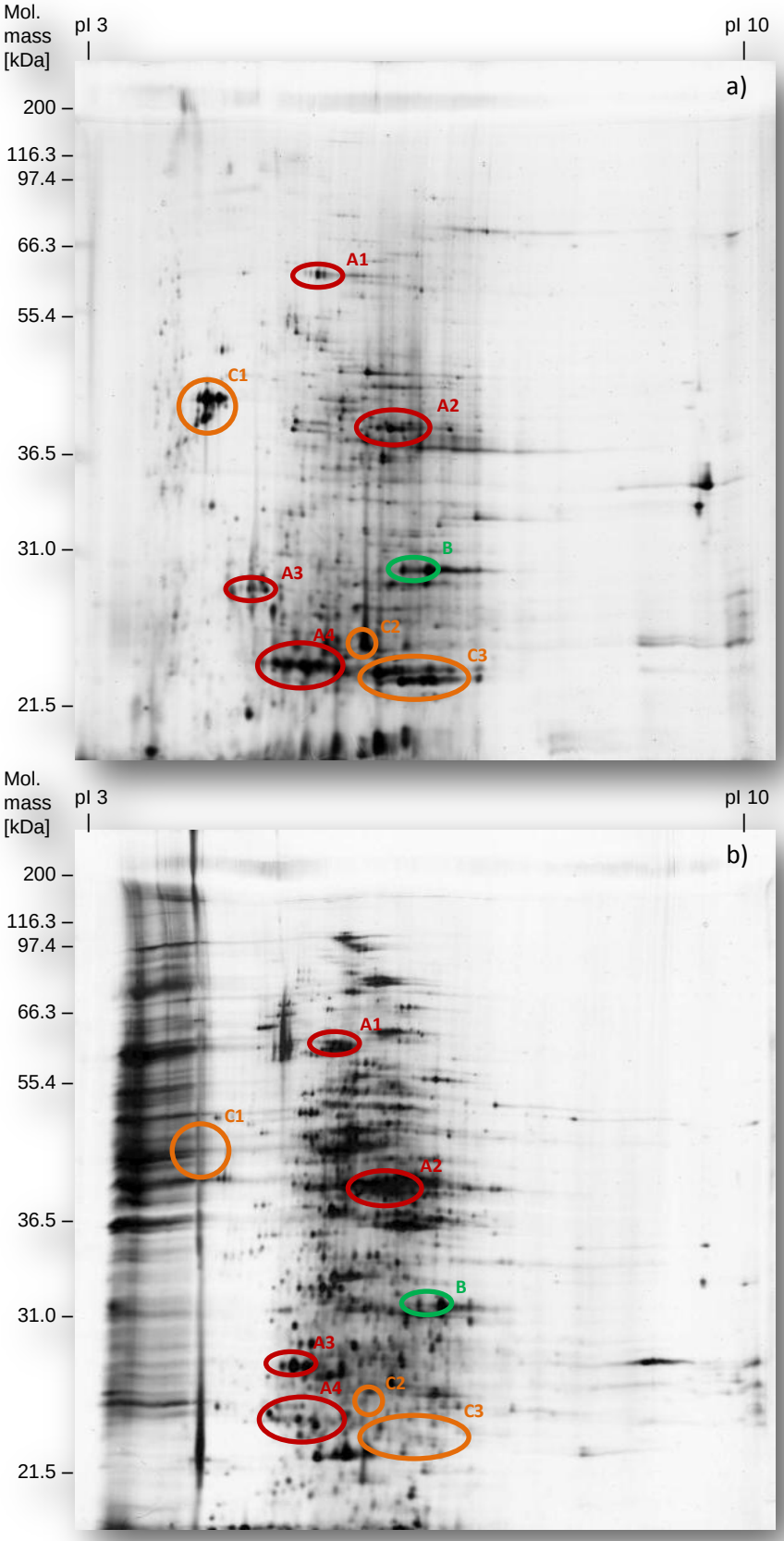

Figure 4.28: 2DE gels of EPS proteins from 14 d-old drinking-water biofilms cultivated at a) plumbing system A, b) distribution system A, c) plumbing system B, d) distribution system B and e) plumbing system C. Encircled in red: spots present in all EPS samples; encircled in green: Benzonase twin-spots; encircled in orange: spot clusters found in plumbing system biofilms only. Protein load: 100 µg.

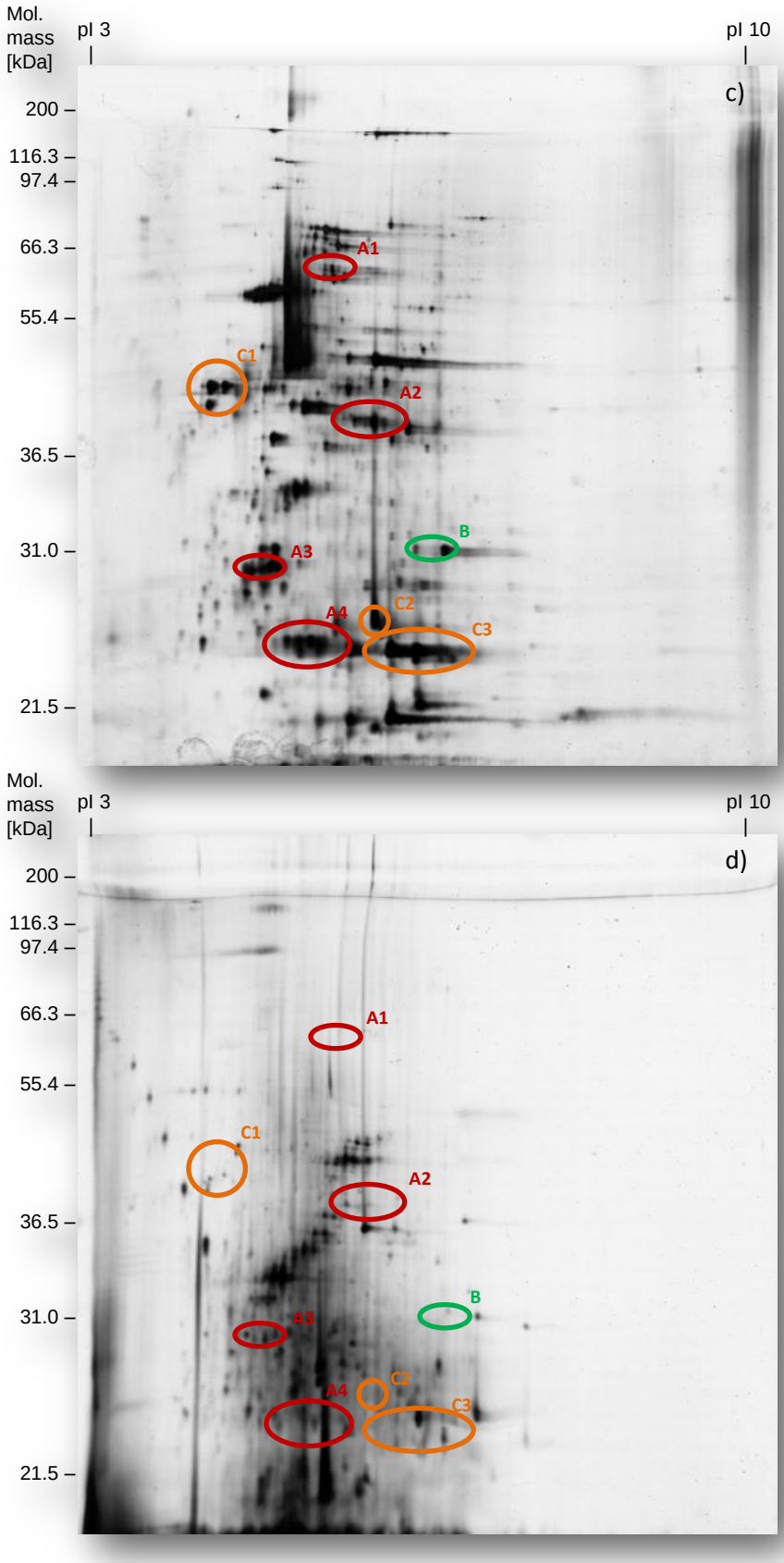

Figure 4.28: *Continued*

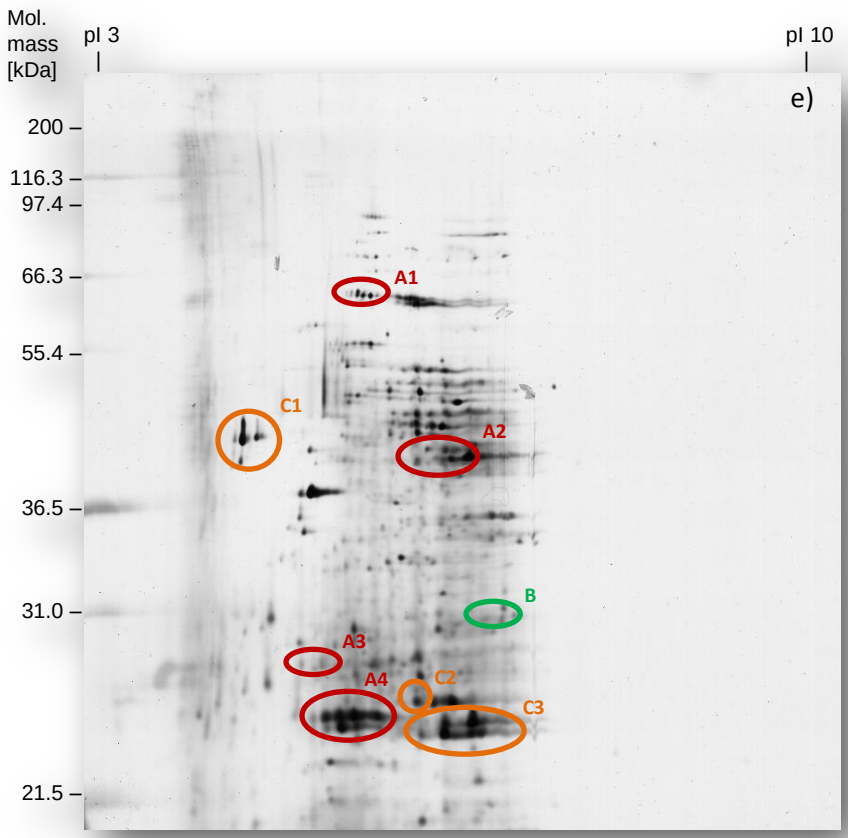

Figure 4.28: *Continued*

4.6 Identification of EPS proteins

The main functions of extracellular proteins are considered to be as enzymes or as structural components of the EPS matrix. To determine the role of EPS proteins in drinking-water biofilms, EPS proteins were separated by 2DE and subjected to identification by MALDI-TOF-MS. For identification of EPS proteins from drinking-water biofilms, the biofilms were cultivated on EPDM for 14 d at plumbing system C and sampled according to sections 3.1 and 3.2. EPS was isolated by 20 min CER treatment (Section 3.8). EPS solutions were incubated with the DNase Benzonase and dialyzed (MWCO 12 – 14 kDa) as clean-up for 2DE, and aliquots containing 400 µg protein were freeze dried. Freeze-dried EPS were subjected to 2DE in duplicates. The EPS were resuspended in IEF buffer and subjected to IEF on IPG strips with a linear pH gradient of 3 to 10 for a total of 95 kVh, followed by separation by size on 12 % SDS polyacrylamide gels. Gels were stained with silver (Blum *et al.*, 1987). A total of 36 protein spots were excised from each gel from same locations on both gels (Fig. 4.29). Proteins present in the excised gel plugs were digested with Trypsin and analyzed by MALDI-TOF-MS.

Identification results are given in Tab. 4.9. The MS-spectra obtained for EPS proteins in most cases showed significant homology (a score ≥ 85 is according to the Mascot software considered significant) to MS-spectra of proteins present in the NCBInr database. The majority of analyzed proteins exhibited significant homology to more than one protein. Molecular weight or pI of the identified proteins only in some cases corresponded to the location of the proteins on the gels. The majority of analyzed EPS protein spots displayed significant homology of MS-spectra to proteins with metabolic, transport, or regulatory functions.

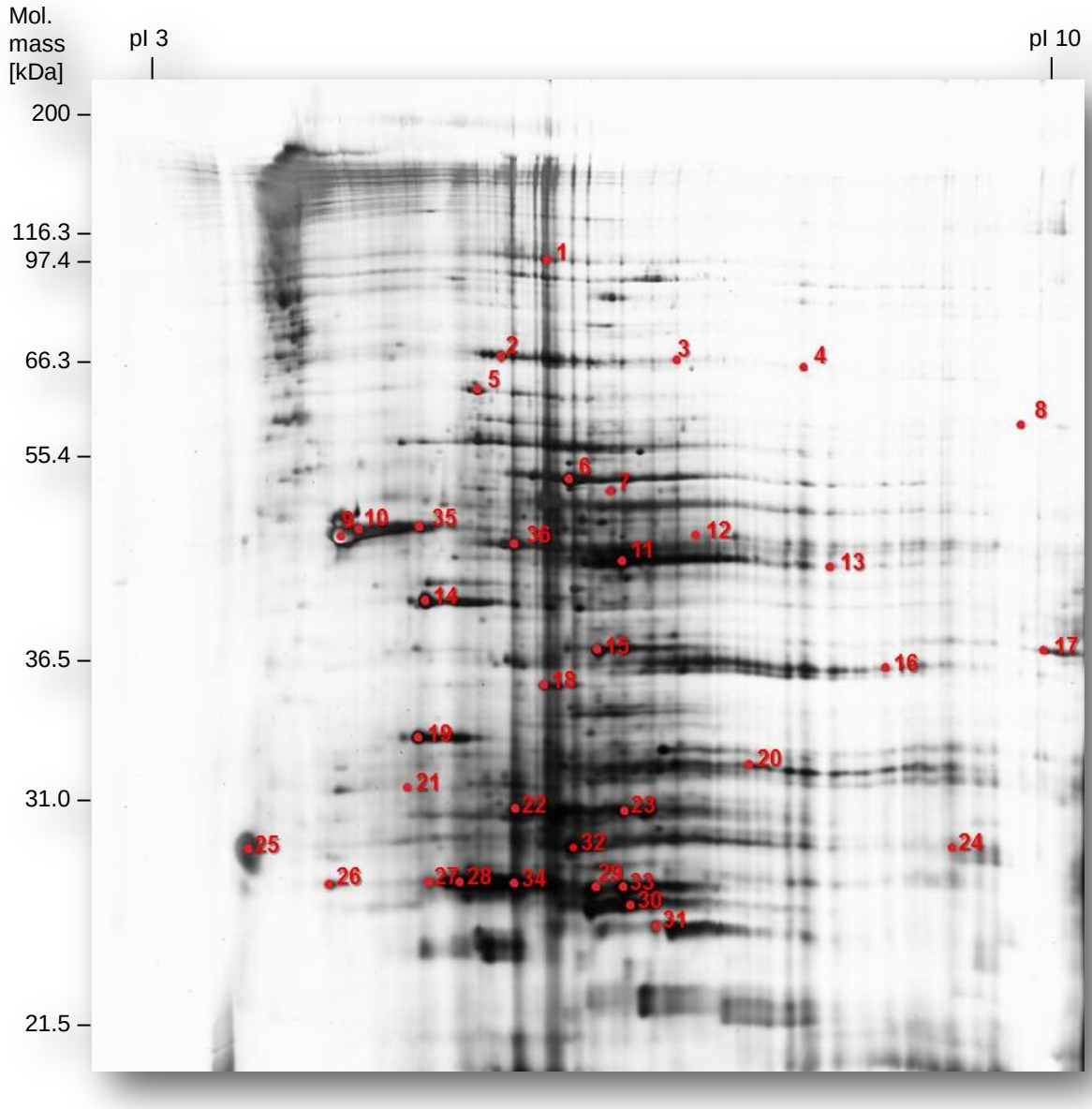

Figure 4.29: 2DE gel of EPS proteins from drinking-water biofilms. Marked spots represent excised protein spots from the 2DE gel. Protein load: 400 µg.

Table 4.9: Identities of EPS proteins of 14 d-old drinking-water biofilms cultivated at plumbing system C. Score ≥ 85 indicates significant homology of mass spectra to identified proteins. Ext, extracellular; OM, outer membrane; P, periplasma; IM, inner membrane; M, membrane; CW, cell wall; Cyt, cytoplasma; Mit, mitochondrial; Nuc, nuclear.

Spot No.	Closest match	NCBI database accession no.	Score	Molecular weight [Da]	pI	Alloca-tion	Function
1	DNA-directed RNA polymerase omega subunit family protein-like protein (*Brachyspira pilosicoli* 95/1000)	YP_003786719	124	648473	4.41	Ext, Cyt	DNA-directed RNA polymerase; lipid binding
	Predicted: nesprin-1-like (*Oreochromis niloticus*)	XP_003449925	91	1000241	5.31	n.d.	Unknown
2	Hypothetical protein MYCTH_2310162 (*Myceliophthora thermophila* ATCC 42464)	AEO60681	92	494309	5.77	Cyt	ATPase activity
	Apolipoprotein A1 / A4 / E (*Brachyspira murdochii* DSM 12563)	YP_003632456	90	891359	4.52	Cyt, Ext	Lipid binding
	Hypothetical protein ANACAC_02896 (*Anaerostipes caccae* DSM 14662)	ZP_02420279	89	631318	4.83	Ext	Unknown
3	PAS/PAC sensor signal transduction histidine kinase (*Syntrophobacter fumaroxidans* MPOB)	YP_847362	87	63326	7.67	Cyt	Two-component sensor
	Putative winged helix family two component transcriptional regulator (*Dyadobacter fermentans* DSM 18053)	YP_003089266	85	33097	8.63	OM, Cyt	DNA binding; response regulator
4	Hypothetical protein LmonocytFSL_12376 (*Listeria monocytogenes* FSL J2-003)	ZP_05298907	87	26866	5.49	n.d.	Unknown
	DEAD / DEAH box helicase domain protein (*Eubacterium cellulosolvens* 6)	ZP_07839880	86	58119	9.95	n.d.	Unknown
5	Recombination activating protein 1 (*Muscisaxicola albilora*)	ACS74334	92	111801	8.78	Cyt, Mit	Acid-amino acid ligase; hydrolase, acting on ester bonds; sequence-specific DNA binding; zinc ion binding
	Predicted protein (*Micromonas pusilla* CMP1545)	XP_003061451	90	614201	4.83	OM	Nucleobase transmembrane transporter; starch binding
	Type I site-specific deoxyribonuclease, HsdR family (*Lactobacillus iners* LactinV 09V1-c)	ZP_07698994	86	132492	5.37	Cyt	ATP-, DNA binding; deoxyribonuclease; helicase
6	Outer membrane protein (*Fusobacterium periodonticum* ATCC 33693)	ZP_06025920	96	368322	8.74	Ext	Autotransporter
	CG41503 (*Drosophila melanogaster*)	NP_001163863	96	38427	9.32	Nuc	Unknown
	Laminin, beta 3 (*Bos Taurus*)	DAA20971	93	132715	6.80	Ext	Structure
	Peptidase M23 (*Muricauda ruestringensis* SDM 13258)	YP_004786771	92	63975	6.01	OM; Ext	Peptidase
7	Predicted: uncharacterized protein LOC100837110 (*Brachypodium distachyon*)	XP_003557657	98	65402	8.58	Nuc	Unknown
	Conserved unknown protein (*Ectocarpus siliculosus*)	CBJ32212	97	191236	5.14	Nuc, cyt	ATP binding; microtubule motor
	Hypothetical protein Mnod_6604 (*Methylobacterium nodulans* ORS 2060)	YP_002501675	91	307019	4.83	OM	Lipid binding/transport
	Hypothetical protein (*Plasmodium vivax* SaI-1)	XP_001613279	86	597652	8.60	Nuc	Dynein complex, microtubule motor
8	Predicted protein (*Populus trichocarpa*)	XP_002329744	80	64789	5.48	Cyt	Mg ion binding; terpene synthase
9	Hypothetical protein Mnod_6604 (*Methylobacterium nodulans* ORS 2060)	YP_002501675	101	307019	4.83	OM	Lipid binding/transport
	RND family efflux transporter MFP subunit (*Methylobacterium radiotolerans* JCM 2831)	YP_001757744	98	44590	6.67	P, OM	Transmembrane transport
	Predicted: nesprin-1-like (*Oreochromis niloticus*)	XP_003443047	98	1009172	5.25	Nuc	Actin binding

Table 4.9: *Continued*

Spot No.	Closest match	NCBI database accession no.	Score	Molecular weight [Da]	pI	Localization	Function
	GD22425 (*Drosophila simulans*)	XP_002078618	92	117556	8.94	Nuc	Unknown
	Hypothetical protein Hbut_1477 (*Hyperthermus butylicus* DSM 5456)	YP_001013646	88	163434	5.59	Cyt	Unknown
	Regulatory protein LuxR (*Sphingomonas wittichii* RW1)	YP_001261431	87	97110	5.29	Cyt	Response regulator
10	Hpothetical protein (*Plasmodium vivax* SaI-1)	XP_001613600	98	517212	6.48	Nuc, Cyt	Unknown
	Hypothetical protein PIIN_07729 (*Piriformospora indica*)	CCA73775	89	112399	9.48	Nuc	Unknown
	Chromosome seggregation Smc protein (*Lactobacillus sakei* subsp. *sakei* 23K)	YP_395323	86	132679	5.29	Cyt	DNA binding
11	Putative MutS domain I (*Clostridium* sp. M62/1)	ZP_06571656	96	337341	5.03	Cyt	ATP binding; helicase activity; methyltransferase activity, mismatched DNA binding
	Hypothetical protein Adeh_3797 (*Anaeromyxobacter dehalogenans* 2CP-C)	YP_467000	94	330833	5.34	OM	Unknown
	Chromosome segregation protein SMC (*Burkholderia pseudomallei* 1710b)	YP_333984	93	140091	5.13	Cyt	ATP binding; DNA binding
12	Hypothetical protein Bphy_5976 (*Burkholderia phymatum* STM815)	YP_001862085	90	64597	9.2	IM	Unknown
	Hypothetical protein E5Q_02672 (*Mixia osmundae* IAM 14324)	GAA96012	88	27174	6.3	P	Hydrolase
13	Hypothetical protein Mnod_6604 (*Methylobacterium nodulans* ORS 2060)	YP_002501675	108	307019	4.83	OM	Lipid binding/transport
	M protein repeat protein (*Arthroderma benhamiae* CBS 112371)	XP_003011730	108	126935	4.93	Cyt, Nuc	Unknown
	Chromosome segregation protein (Psc1), putative (*Trichophyton verrucosum* HKI 0517)	XP_003018785	101	126950	4.96	Nuc, Cyt	Unknown
	M protein repeat-containing protein (*Trichophyton equinum* CBS 127.97)	EGE07815	90	126952	4.95	Nuc, Cyt	Unknown
	Predicted protein (*Trichoderma reesei* QM6a)	EGR47626	87	249607	5.07	Nuc, Cyt	Helicase; Hydrolase
	Hypothetical protein SKA34_20542 (*Photobacterium* sp. SKA34)	ZP_01159900	87	228149	4.70	Ext	Unknown
	Hypothetical protein TRIVIDRAFT_86434 (T*richoderma virens* Gv29-8)	EHK26410	86	249714	5.05	Nuc, Cyt	Helicase; Hydrolase
14	Ubiquitin ligase subunit CulD (*Metarhizium acridum* CQMa 102)	EFY91384	108	95784	9.04	Nuc, Mit	Ligase
	Putative lipoprotein (*Streptococcus parasanguinis* SK236)	EGU65582	87	14305	6.73	M, Ext	Unknown
	Hypothetical protein MYCTH_2307003 (*Myceliophthora thermophila* ATCC 42464)	AEO59067	86	126011	5.03	Nuc, Cyt	Unknown
15	DNA-directed RNA polymerase omega subunit family protein-like protein (*Brachyspira pilosicoli* 95/1000)	YP_003786719	88	648473	4.41	Ext, Cyt	DNA-directed RNA polymerase; lipid binding
	Polysaccharide pyruvyl transferase (*Streptomyces lividans* TK24)	ZP_06532812	87	42955	5.85	Cyt	Transferase
	Hypothetical protein SCO0893 (*Streptomyces coelicolor* A3 (2))	NP_625192	86	44345	5.94	Cyt	Transferase

Table 4.9: *Continued*

Spot No.	Closest match	NCBI database accession no.	Score	Molecular weight [Da]	pI	Localization	Function
16	Hypothetical protein MexAM1_META1p4913 (*Methylobacterium extorquens* AM1)	YP_002965797	97	296899	4.90	OM	Lipid binding/transport
	Nucleotide sugar dehydrogenase (*Thermus thermophilus* SG0.5JP17-16)	AEG33386	89	50719	5.87	Cyt	NAD binding; UDP-glucose 6-dehydrogenase
	Hypothetical protein Mext_4468 (*Methylobacterium extorquens* PA1)	YP_001641907	86	296904	4.90	OM	Lipid binding/transport
17	Predicted protein (*Populus trichocarpa*)	XP_002317754	84	38438	7.79	Mit	Metal ion binding; oxidoreductase
	Hypothetical protein PTT_09948 (*Pyrenophora teres* f. *teres* 0-1)	XP_003299037	82	163779	6.27	Nuc	Unknown
18	Type III effector protein XopAD (*Xanthomonas oryzae* pv. *oryzicola* BLS256)	AEQ98548	93	314948	7.65	P, OM	Binding
	Hypothetical protein HMPREF9413_4085 (*Paenibacillus* sp. HGF7)	ZP_08507129	92	242936	5.69	Cyt	Nucleoside-triphosphatease; nucleotide binding
	CG3173 (*Drosophila melanogaster*)	NP_611875	91	237286	6.15	Nuc	Binding
	CD05534p (*Drosophila melanogaster*)	AAN71641	91	237314	6.15	Nuc	Binding
	Hypothetical protein MTES_1762 (*Microbacterium testaceum* StLB037	YP_004224606	91	31853	8.24	Cyt	Unknown
	Ppx / GppA phosphatase (*Hyphomicrobium denitrificans* ATCC 51888)	YP_003756958	90	56501	9.00	Cyt	Phosphatase
	Nonstructural polyprotein (*Microbacterium testaceum* StLB037)	YP_004224606	90	279067	6.75	Virus	ATP-, RNA binding; RNA polymerase; cysteine-type peptidase; helicase; mRNA methyltransferase
	Nonstructural polyprotein (Chikungunya virus)	ADG95951	89	279126	6.90	Virus	Helicase; hydrolase; methyltransferase; nucleotidyltransferase; protease; RNA polymerase; thiol protease; transferase
	GE14364 (*Drosophila yakuba*)	XP_002092752	88	237110	6.27	Nuc	Binding
	Hypothetical protein CLF_101160 (*Clonorchis sinensis*)	GAA48087	87	38687	8.95	Cyt	Ca ion binding
	Biopolymer transport protein ExbD/TolR (*Bacteroides helcogenes* P 36-108)	YP_004160702	86	24450	5.76	P	Transmembrane protein transport
	GA module protein (*Gardnerella vaginalis* 315-A)	EGL13251	85	213357	5.77	P	Pathogenesis
19	Hypothetical protein DespoDRAFT 1881(*Desulfobacter postgatei* 2ac9)	ZP_09097030	92	13368	5.02	n.d.	Unknown
	Nitrate reductase [NAD(P)H], large subunit (*Staphylococcus aureus* A9635)	ZP_05687927	87	89608	5.28	Cyt	Oxidoreductase
20	Hypothetical protein bthur0013_51610 (*Bacillus thuringiensis* IBL 200)	ZP_04074828	98	20510	8.67	Cyt, Ext	Unknown
	Thrombospondin type 3 repeat superfamily protein (*Peptoniphilus duerdenii* ATCC BAA-1640)	ZP_07400466	92	323865	5.11	CW	Ca binding
21	Hypothetical protein NEMVEDRAFT_v1g222052 (*Nematostella vectensis*)	XP_001621378	72	23441	4.33	Cyt, Nuc	Unknown
22	Putative methylase (*Streptomyces hygroscopicus* ATCC 53653)	ZP_07293066	86	23036	10.8	M	Methyltransferase

Table 4.9: *Continued*

Spot No.	Closest match	NCBI database accession no.	Score	Molecular weight [Da]	pI	Localization	Function
23	Hypothetical protein PGTG_18071 (*Puccinia graminis* f. sp. *tritici* CRL 75-36-700-3)	XP_003336296	89	200358	6.85	Nuc	Binding
	L-proline dehydrogenase (*Intrasporangium calvum* DSM 43043)	YP_004097913	87	34825	5.92	Cyt	Proline dehydrogenase
	Type III effector protein XopAD (*Xanthomonas oryzae* pv. *oryzicola* BLS256)	AEQ98548	86	314948	7.65	P, OM	Binding
24	Transcriptional regulator (*Streptomyces clavuligerus* ATCC 27064)	ZP_05005765	87	76376	8.68	Cyt	ADP-, DNA binding; Nucleoside-triphosphatase; response regulator
	Hypothetical protein MICPUN_96907 (*Micromonas* sp. RCC299)	XP_002507402	87	100369	5.87	Chl	ATP binding; kinase; pyruvate, phosphate dikinase
	Diguanylate cyclase/phosphodiesterase (*Acetivibrio cellulolyticus* CD2)	ZP_07327785	85	102667	5.55	n.d	Unknown
25	Hypothetical protein zobellia_3150 (*Zobellia galactanivorans*)	YP_004737568	96	79410	5.69	Cyt	Catalytic activity
26	Predicted protein (*Micromonas* sp. RCC299)	XP_002502059	91	36333	9.87	Mit, Cyt	Oxidoreductase
27	PAS/PAC sensor signal transduction histidine kinase (*Ktedonobacter racemifer* DSM 44963)	ZP_06967808	100	63582	4.99	Cyt	Two-component sensor
	AAA ATPase, central domain protein (*Thermosinus carboxydivorans* Nor1)	ZP_01666434	91	38495	6.63	Cyt	Nucleoside-triphosphatase
	Outer membrane component of multidrug efflux pump (*Xanthomonas fuscans* subsp. *aurantifolii* str. ICPB 10535)	ZP_06731747	88	51074	6.08	OM	Transport
	Eukaryotic translation initiation factor 2c (*Ajellomyces capsulatus* H143)	EER37077	86	100942	9.65	Mit	Translation initiation factor
28	YaeB (*Klebsiella pneumonia*)	YP_003517426	86	10635	7.90	Cyt	Unknown
	alanyl-tRNA synthetase (*Pseudomonas putida* W619)	YP_001750614	85	97369	5.36	Cyt	ATP binding; alanine-tRNA ligase; metal ion binding; tRNA binding
29	Conserved hypothetical protein (*Lactobacillus brevis* subsp. *gravesensis* ATCC)	ZP_03939650	106	310886	5.30	Ext, M	Cell wall component
	DENN domain-containing protein (*Arthroderma otae* CBS 113480)	XP_002844092	90	104116	6.53	Nuc	Unknown
30	Hypothetical protein BMS_1974 (*Bacteriovorax marinus* SJ)	CBW26789	88	124459	5.3	Ext, OM	Unknown
31	RmuC domain protein (*Neisseria subflava* NJ9703)	ZP_05985826	96	68119	5.62	OM	Unknown
	FrnA (*Streptomyces roseofulvus*)	AAC18096	90	83322	8.94	Cyt	Transporter
32	Putative two-component response regulator (Uncultured bacterium)*	Q6JWS4	179	23727	5.39	Cyt	Response regulator
	Two component LuxR family transcriptional regulator (*Acidovorax* sp. JS42)	YP_986372	126	23196	5.51	Cyt	Response regulator
33	Predicted protein (*Nematostella vectensis*)	XP_001640716	109	104766	4.96	Nuc	Unknown
	Myelin transcription factor 1-like protein (*Rattus norvegicus*)	NP_446340	95	134793	4.83	Nuc	RNA polymerase II transcription activator; retinoic acid-responsive element binding; sequence-specific DNA binding transcription factor; zinc binding
	Hypothetical protein HMPREF933_02224 (*Johnsonella ignava* ATCC 51276)	ZP_09155338	88	399796	5.15	Cyt, OM	ATP binding; DNA binding, helicase activity, methyltransferase activity

Table 4.9: *Continued*

Spot No.	Closest match	NCBI database accession no.	Score	Molecular weight [Da]	pI	Localization	Function
34	Hypothetical protein DAPPUDRAFT_111031 (*Daphnia pulex*)	EFX72104	95	293681	7.91	Nuc, Cyt	Binding
	Xaa-Pro aminopeptidase (*Microscilla marina* ATCC 23134)	ZP_01688987	89	55600	9.35	P	Aminopeptidase
	Type III effector HopR1 (*Pseudomonas syringae* pv. *aesculi* str. 2250)	ZP_06479893	87	203281	6.13	OM	Unknown
	BNI1 (*Saccharomyces cerevisiae*)	CAA63225	87	174900	6.13	Nuc	Binding
35	Protein unc-22 (*Brugia malayi*)	XP_001899339	91	759185	5.61	Nuc, Cyt	Protein kinase
	Exonuclease SbcC (*Borrelia burgdorferi* 94a)	ZP_03770352	86	111868	8.24	OM; Cyt	Exonuclease
36	Succinyl-CoA synthetase subunit beta (*Polaromonas* sp. JS666)	YP_551463	93	41409	5.21	Cyt	ATP binding; metal ion binding; succinate-CoA ligase

*detected in drinking-water biofilm (Stoeckigt et al., unpublished).

4.7 Influence of *Pseudomonas aeruginosa* on drinking-water biofilm composition

Recent studies have demonstrated the potential of drinking-water biofilms to accommodate hygienically relevant microorganisms, which may pose a threat to human health (Szewzyk *et al.*, 2000; Flemming *et al.*, 2002; Bressler *et al.*, 2009; Moritz *et al.* 2010*). P. aeruginosa* is known to occasionally occur as a contaminant of drinking-water biofilms and represents such a hygienically relevant organism due to its facultative pathogenic character. The aim of the present study was the investigation of the colonization of established drinking-water biofilms with *P. aeruginosa* to determine, whether changes to the biofilm composition and properties in presence of this organism occur. Drinking-water biofilms were grown on EPDM coupons as described in section 3.1 in parallel in two separate biofilm reactors connected to plumbing system C. After 14 d of biofilm growth the water flow was stopped, 3 EPDM coupons were aseptically removed from each biofilm reactor and biofilms were sampled according to section 3.2 to obtain information on the biofilms prior to inoculation. One of the biofilm reactors was inoculated with *P. aeruginosa* AdS in deionized water at a concentration of 1 x 10^6 cell mL^{-1} and the biofilms were statically incubated for 24 h (+ P.a.). The other reactor was inoculated with deionized water only and served as negative control (- P.a. control). After the incorporation drinking water flow was restarted and biofilms were sampled after additional 24 h or 7 d of cultivation at flow conditions. The biofilms were analyzed by microbiological, biochemical, or molecular biological means for potential alterations of the EPS matrix caused by *P. aeruginosa*. Results represent the arithmetic means ± standard deviation of two independent biofilm cultivation runs.

4.7.1 Incorporation of *P. aeruginosa* into drinking-water biofilms

Drinking-water biofilms were analyzed for total cell count, HPC, as well as presence of *P. aeruginosa* AdS within drinking-water biofilms by cultivation on CN agar or the cultivation-independent FISH. All parameters were determined in drinking-water biofilms prior to incorporation of *P. aeruginosa* AdS (biofilm age: 14 d), 24 h after incorporation (biofilm age: 16 d), or 7 d after incorporation (biofilm age: 22 d).

All biofilms showed relatively similar total cell counts in drinking-water biofilms in the range of 2.4 x 10^8 ± 1.8 x 10^7 and 4.9 x 10^8 ± 2.6 x 10^8 cells cm^{-2} (Fig. 4.30). Culturability of biofilm cells on R2A medium exhibited a slightly increasing trend throughout the experimental run, and was in the range of 3.1 x 10^6 ± 2.9 x 10^6 cfu cm^{-2} and 1.4 x 10^8 ± 8.3 x 10^7 cfu cm^{-2}.

Presence of *P. aeruginosa* in drinking-water biofilms prior to inoculation was not detected by cultivation on CN agar, which is selective for *P. aeruginosa*, or by FISH. Cultivation on CN agar, furthermore, did not detect *P. aeruginosa* in any biofilms, irrespectively of the addition of *P. aeruginosa* in the inoculum or the duration of cultivation (detection limit: 0.5 cfu cm^{-2}). Determination of *P. aeruginosa* by FISH, on the other hand, revealed 1.2 x 10^5 ± 5.9 x 10^4 cells cm^{-2} 24 h after inoculation and 2.6 x 10^5 ± 2.8 x 10^5 cells cm^{-2} 7 d after inoculation. Presence of *P. aeruginosa* in negative control biofilms 24 h or 7 d after inoculation with sterile deionized water was not detected by FISH.

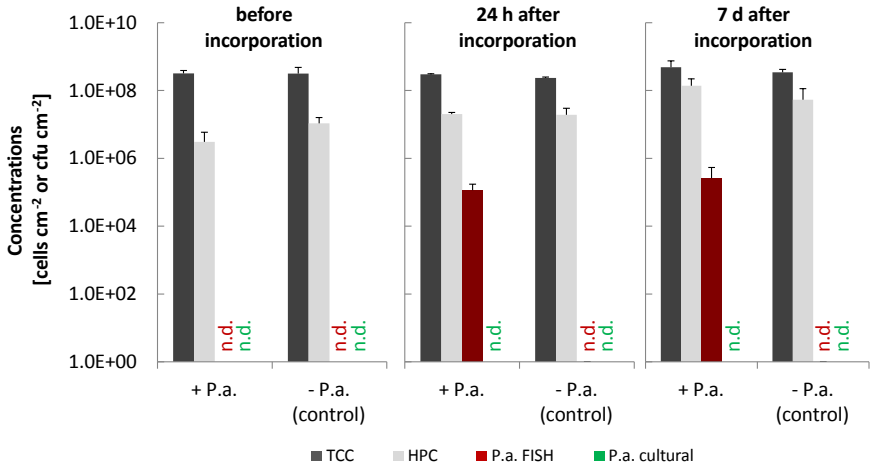

Figure 4.30: Total cell count and HPC of drinking-water biofilms and concentrations of *P. aeruginosa* AdS before as well as 24 h and 7 d after inoculation. Total cell count was determined by DAPI staining, HPC was analyzed by spread-plate method on R2A medium and enumeration of colonies after 7 d incubation at 20 °C. *P. aeruginosa* AdS concentrations were determined by FISH using the Psae16S-182 probe as well as culturally by spread-plate method on CN agar. + P.a. indicates biofilms used for inoculation *with P. aeruginosa* in deionized water, - P.a. (control) indicates negative control biofilms used for inoculation with deionized water only. n.d., not detected; n = 2 independent cultivation runs.

A discrepancy between detection of *P. aeruginosa* by cultivation on CN agar and FISH was evident. A potential reason for the difference could be the loss of culturability of *P. aeruginosa* due to Cu ions present in the drinking water used for biofilm cultivation. Cu at concentrations of 0.14 mg L^{-1}, as present in drinking water from plumbing system C, has been shown to cause a transition of *P. aeruginosa* into the VBNC state (Dwidjosiswojo *et al.*,

2011). Hence, the effect of drinking water as well as Cu ion solutions on *P. aeruginosa* was investigated.

P. aeruginosa AdS pure cultures were exposed to different dilutions of drinking water in deionized water for 24 h. After incubation the suspensions were analyzed for total cell number and culturability on nutrient agar. Enumeration of cells displayed similar total cell counts in all dilutions of drinking water, ranging from 1.7 x 10^6 to 2.5 x 10^6 cells mL^{-1} (Fig. 4.31a). Cultivation of *P. aeruginosa* in the inoculum (deionized water control, 0 % drinking water) showed colony counts comparable to the total cell counts of 1.3 x 10^6 cfu mL^{-1}. Exposure of *P. aeruginosa* to drinking water resulted in significant reduction of culturability by 5 orders of magnitude already in 5 fold diluted drinking water, which corresponded to a 0.44 µM Cu content.

P. aeruginosa AdS pure cultures were, furthermore, exposed to different concentrations of Cu in 6 mM phosphate buffer at pH 7.0 for 24 h and subsequently analyzed for total cell count and culturability on nutrient agar. Total cell counts were in the range of 3.3 x 10^6 ± 1.9 x 10^5 cells mL^{-1} and 4.4 x 10^6 ± 3.2 x 10^5 cells mL^{-1} (Fig. 4.31b). Culturability of *P. aeruginosa* exposed to Cu in concentrations between 0 µM and 0.6 µM was marginally lower compared to total cell counts, revealing colony numbers between 1.1 x 10^6 ± 8.7 x 10^4 cfu mL^{-1} and 1.5 x 10^6 ± 6.1 x 10^5 cfu mL^{-1}. Cu concentrations > 0.6 µM caused a decrease of culturability with increasing Cu content, showing 9.3 ± 5.7 cfu mL^{-1} in 4 µM Cu concentrations.

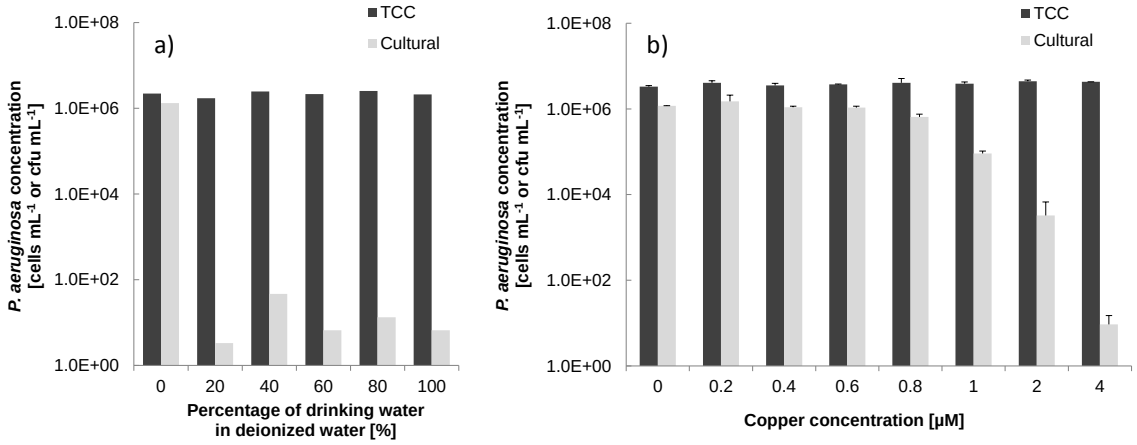

Figure 4.31: Total cell count (TCC) and culturability of *P. aeruginosa* AdS after 24 h exposition at 20 °C to a) dilutions of drinking water in deionized water (n = 1), or b) different concentrations of copper in 6 mM phosphate buffer at pH 7.0 (n = 2). Total cell count was determined by DAPI-staining, culturability was analyzed by spread-plate method on nutrient agar.

4.7.2 Influence of *P. aeruginosa* on the biochemical composition of drinking-water biofilms

Drinking-water biofilms and their EPS matrix after incorporation of *P. aeruginosa* were analyzed for protein, carbohydrate and eDNA content. Drinking-water biofilms inoculated with *P. aeruginosa* AdS as well as isolated EPS exhibited slightly lower quantities of proteins, carbohydrates and DNA compared to the concentrations of the control biofilms of the respective age (Fig. 4.32). The major difference was detected in extracellular DNA concentrations, which were 50 % (24 h after incorporation) and 31 % (7 d after incorporation) lower, respectively, compared to the control biofilms of the respective age.

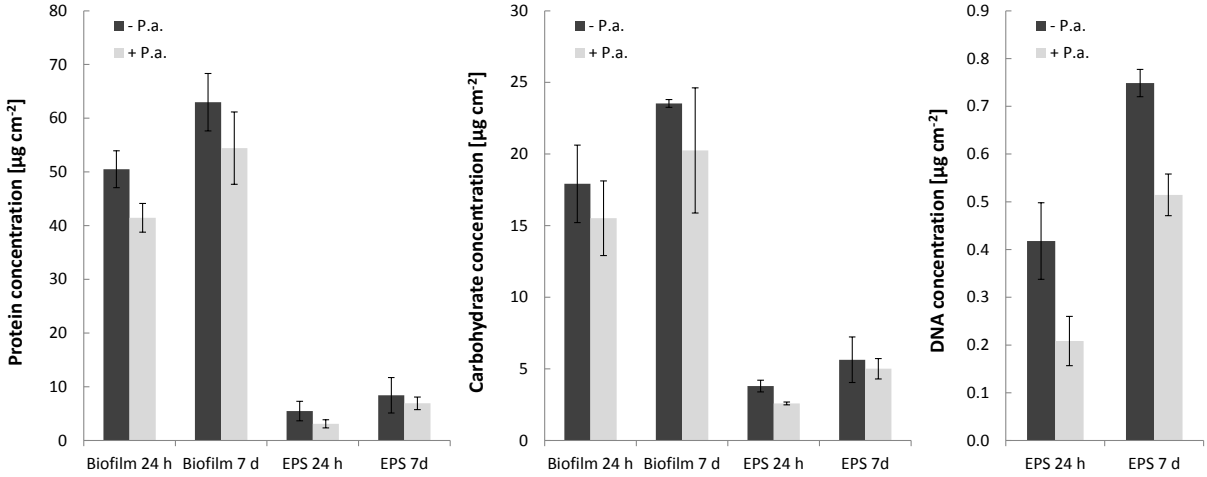

Figure 4.32: Protein, carbohydrate and eDNA concentrations in drinking-water biofilms and isolated EPS, 24 h or 7 d after inoculation with *P. aeruginosa* AdS (+P.a.) or deionized water (-P.a. control). Proteins were quantified by the modified Lowry assay (Peterson, 1977), carbohydrates were determined by the sulphuric acid/phenol method, eDNA was analyzed by PicoGreen (n = 2 independent biofilm cultivation runs).

Drinking-water biofilms were qualitatively analyzed by 2DE to determine qualitative changes of extracellular proteins caused by the incorporation of *P. aeruginosa* into the biofilms. Protein spot patterns were produced for EPS isolated from drinking-water biofilms prior to incorporation of *P. aeruginosa* (biofilm age: 14 d), and 24 h (biofilm age: 16 d) or 7 d after incorporation (biofilm age: 22 d), as well as for the respective negative controls.

2DE of extracellular protein resulted in spot patterns showing between 450 and 500 spots in the range of pH 3 to 10 and molecular weights of 20 to 200 kDa prior to incorporation of *P. aeruginosa*, as well as 24 h or 7 d after incorporation (Fig. 4.33). The spot patterns of EPS isolated from drinking-water biofilms before inoculation, 24 h after inoculation with

P. aeruginosa, and the control 24 h after inoculation with deionized water showed slight variations of the spot profiles, however, no significant changes were evident. Prolonged incubation resulted in a smaller number of high molecular weight proteins as well as basic proteins 7 d after inoculation with *P. aeruginosa* or the control. This decrease was similar to the results obtained in the dynamics studies in section 4.4.4. EPS before inoculation or 24 h after inoculation showed 35 to 40 high molecular weight spots > 66 kDa. These spots were not detected in the EPS of drinking-water biofilms 7 d after incorporation, which showed protein spots mainly in pH regions between pH 4 and 7 and molecular weights of 21.5 and 66.3 kDa.

Comparison of extracellular protein spot patterns of biofilms 7 d after inoculation with *P. aeruginosa* in deionized water with the control biofilm inoculated only with deionized water revealed a small number of noticable alterations to the extracellular protein pattern (Fig. 4.33d,e). Region 1 displayed a cluster of proteins composed of approximately 10 spots, which was present in biofilms before inoculation, 24 h after inoculation with *P. aeruginosa* and its control, and 7 d after inoculation with *P. aeruginosa*. This cluster, however, was missing in the EPS of negative control biofilms 7 d after inoculation. Regions 2 and 3 on the other hand showed clusters of proteins, each composed of 5 spots, which are only present in the EPS of negative control biofilms 7 d after inoculation, but in no other sample.

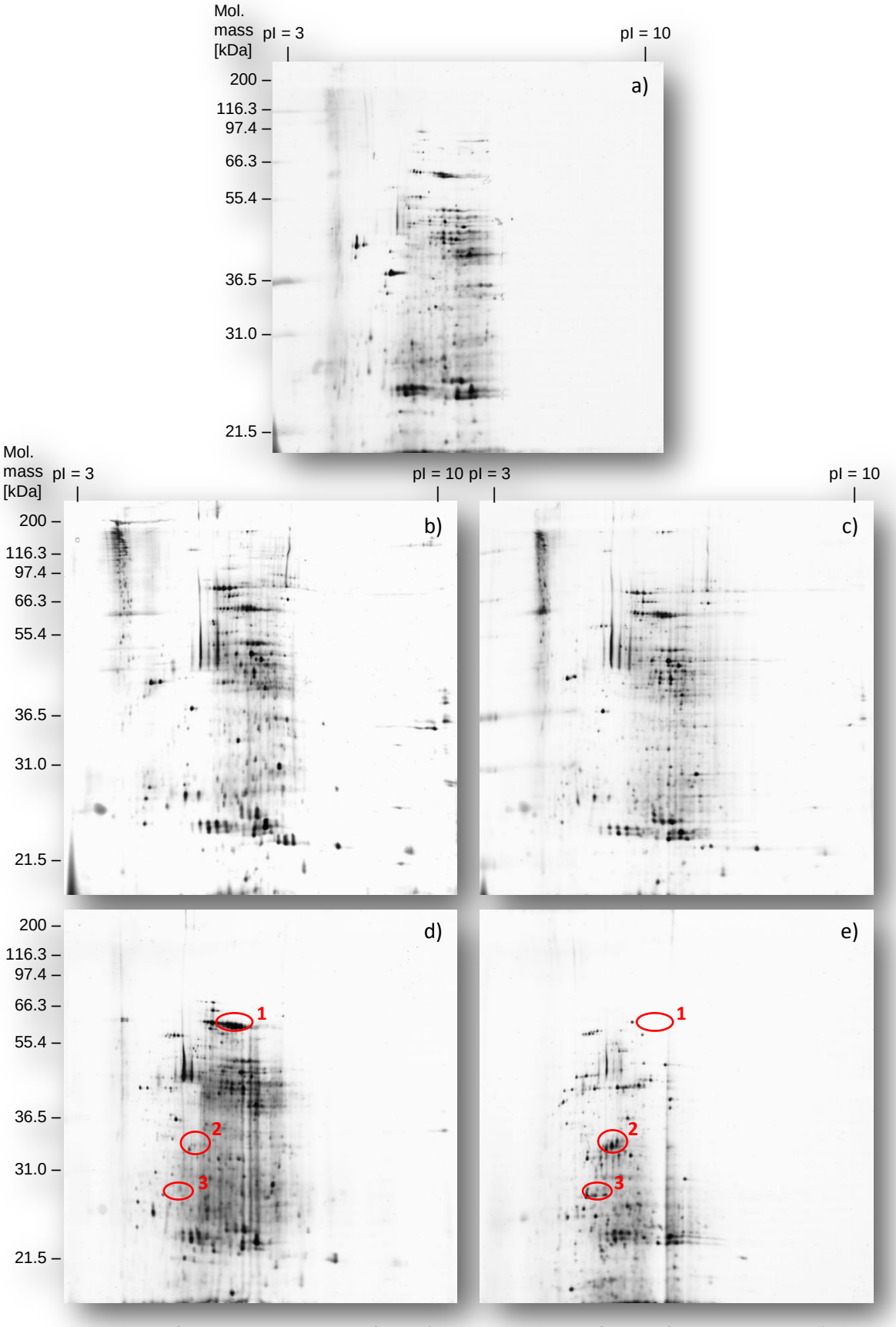

Figure 4.33: 2DE gels of EPS proteins isolated from a) drinking-water biofilms before inoculation, b) drinking-water biofilm 24 h after inoculation with *P. aeruginosa* AdS in deionized water, c) drinking-water biofilm 24 h after inoculation with deionized water (control), d) drinking-water biofilm 7 d after inoculation with *P. aeruginosa* AdS in deionized water, and e) drinking-water biofilm 7 d after inoculation with deionized water (control). Encircled in red: regions of major differences in spot patterns.

5. DISCUSSION

Drinking-water biofilms are ubiquitous in drinking water distribution as well as plumbing systems. Generally, drinking-water biofilms are formed by autochthonous microorganisms, which are considered harmless to human health (Wingender, 2011; Flemming *et al.*, 2012). The oligotrophic character of drinking water permits only limited biofilm development, however, once nutrients become available, extensive growth can occur, leading to possible contamination of the water phase. Furthermore, recent studies have demonstrated the potential of drinking-water biofilms to accommodate hygienically relevant microorganisms, which may pose a threat to human health (Szewzyk *et al.*, 2000; Flemming *et al.*, 2002; Bressler *et al.*, 2009; Moritz *et al.* 2010). This signifies the need of research in the field of biofilm development and composition in drinking water systems, in order to understand the formation mechanisms in this kind of environment and the biofilms' significance as reservoirs for hygienically relevant microorganisms. Despite numerous studies investigating biofilm formation in these systems, none has addressed the EPS composition and function of EPS within drinking-water biofilms.

The aim of the current study was the characterization of drinking-water biofilms with particular regard to their EPS. Knowledge of the composition of drinking-water biofilms and their EPS is essential, since EPS can be important targets for measures aimed at the inactivation and removal of biofilm organisms from technical systems. In the first part of the study, methods for cultivation of drinking-water biofilms, for EPS isolation and for analysis were established and adapted, which was a particular challenge, due to the low amounts of biomass usually found in drinking water systems. Drinking-water biofilms were cultivated on coupons made of an EPDM rubber material, which met physical and chemical requirements of the German KTW guideline (Umweltbundesamt, 2011) for plastic materials in drinking

water, but not the microbiological recommendations according to the technical rule W270 of the German Water and Gas Association (DVGW, 2007). This material was shown to support elevated growth of drinking-water biofilms in real drinking water systems, causing continuous contamination of the water phase (Kilb *et al.*, 2003). Also Bressler *et al.* (2009) indicated elevated drinking-water biofilm formation on this type of elastomer in a similar experimental setup as used in the current study. The extensive biofilm formation on this elastomer demonstrates a worst-case scenario for drinking water systems. This material was applied in this study to obtain sufficient biomass for the analysis of drinking-water biofilms and their EPS.

The characterization of EPS from drinking-water biofilms required isolation of EPS from the biofilm. There is no universal method for the isolation of EPS from biofilms. Hence, the aim was to establish a method for EPS isolation from drinking-water biofilms able to recover high yields of EPS and allowing for a broad range of analytical methods for EPS characterization. Commonly applied EPS isolation methods were tested and compared for their suitability to isolate EPS from drinking-water biofilms. The methods were evaluated based on their EPS yield, detrimental impact on biofilm cells, as well as compatibility with subsequent analytical methods. The focus was on the CER isolation method described by Jahn and Nielsen (1995), which was miniaturized and optimized in the present study.

The established methods for cultivation, sampling and characterization of drinking-water biofilms and EPS were applied to investigate different aspects of drinking-water biofilms. The progress of biofilm formation on EPDM was analyzed, to obtain information on the dynamics of drinking-water biofilms and their EPS throughout the different stages of biofilm growth. Moreover, drinking-water biofilms were cultivated on EPDM in different distribution and copper plumbing systems to examine the influence of drinking water characteristics as well as plumbing materials on biofilm and EPS composition, and to investigate variability among drinking-water biofilms according to their origin. Furthermore, the potential of drinking-water biofilms to harbor hygienically relevant microorganisms was addressed. Established drinking-water biofilms were inoculated with *P. aeruginosa* as a model microorganism of hygienical relevance, to examine its incorporation, persistence and influence on drinking-water biofilm and EPS composition.

The EPS matrix, which mainly consists of polysaccharides, proteins, DNA, and lipids, has a profound role for the establishment and maintenance of biofilms (Flemming and Wingender, 2010). Extracellular proteins are considered to have their major functions as enzymes or as structural components of biofilms (Wingender and Jaeger, 2002). However, identification of extracellular enzymes within biofilms has only been attempted in a small number of studies. In the present study, extracellular proteins were of particular interest and were analyzed and identified to determine their function in the EPS matrix of drinking-water biofilms.

5.1 EPS isolation from drinking-water biofilms

EPS isolation is generally the most crucial step for the analysis of EPS, as the efficiency and completeness of the isolation method determines the outcome of any subsequent analysis. There is no universal method for the isolation of EPS, instead, various physical and chemical methods have been developed in past decades, aiming at the destabilization and consequential solubilization of EPS components. In this study EPS isolation from drinking-water biofilms was pursued, in order to quantitatively and qualitatively analyze EPS composition. EPS isolation from drinking-water biofilms represents a challenge due to the usually low amounts of biomass found on surfaces of drinking-water systems. The requirements for the EPS isolation method in this case were a) the method should mobilize sufficient amounts of EPS components from the drinking-water biofilm matrix, b) the method should allow for a broad range of subsequent analytical methods without interference for a thorough characterization of the biofilms, and c) the isolation treatment should not affect biofilm cell integrity. In this study the main focus was on EPS isolation by CER (Jahn and Nielsen, 1995; Frølund *et al.*, 1996), which has shown very promising results in a study conducted by Bressler (2008) for EPS isolation from drinking-water biofilms. The CER isolation was miniaturized, to meet the requirements of the low biomass of drinking-water biofilms, and optimized, in respect to duration of shaking with CER. The optimized CER isolation was compared to four alternative EPS isolation procedures conventionally applied for activated sludge or pure culture biofilms, which included shaking (control), as well as stirring in presence of formaldehyde and NaOH, stirring in presence of EDTA and heating.

The substratum EPDM and the cultivation conditions allowed for the growth of relatively thick biofilms of drinking-water organisms, so that a sufficient amount of EPS was present within the biofilms and could be recovered in quantifiable amounts by all isolation methods. The EPS yields varied depending on the EPS isolation method. Furthermore, the addition of chemicals, such as formaldehyde, NaOH, or EDTA, for EPS isolation from drinking-water biofilms showed limitations for subsequent EPS analyses (Tab. 5.1).

- CER isolation -

CER treatment has been applied for EPS isolation from a variety of biofilms, including pure culture biofilms of several microbial species, sewer biofilms, activated sludge biofilms and benthic biofilms (Tab. 1.2). This method functions through the mobilization of multivalent cations which cross-link EPS polymers, resulting in the destabilization of the EPS matrix and enhanced solubilization of EPS components (Rudd *et al.*, 1983; Jahn and Nielsen, 1995; Frølund *et al.*, 1996). The selectivity of the CER towards divalent cations has been described by Bonner and Smith (1956), using the CER Dowex 50, and showing the following hierarchy: Ba > Pb > Sr > Ca > Ni > Cd > Cu > Co > Zn > Mg. Presence of multivalent cations including Ca^{2+} and Mg^{2+}, which are characteristic cations in drinking water, as well as $Fe^{2+/3+}$, $Cu^{1+/2+}$, Zn^{2+} and Al^{3+}, originating from the distribution system or plumbing materials, was confirmed in drinking-water biofilms in the current study. These cations were hypothesized to stabilize drinking-water biofilm's EPS matrix. Treatment of drinking-water biofilms with CER was expected to bind these cations and liberate increased amounts of EPS components also in this type of biofilm analyzed in the present study.

The isolation of EPS by use of the CER proved to be most suitable for the recovery of EPS from drinking-water biofilms in terms of EPS yields, without causing damage to biofilm cells or interference with subsequent analyses. Significantly higher EPS yields were isolated with the help of the CER compared to simple shaking treatment. Similar results were obtained by Jahn and Nielsen (1995) and Frølund *et al.* (1996) for EPS isolated from sewer biofilms and activated sludge, respectively. There was no interference with any subsequent analyses, as the CER was completely separated from the treated biofilm suspension by simple sedimentation. Also, absence of activity of the strictly intracellular enzyme G6PDH in the EPS

solutions indicated no cell lysis induced by CER treatment for up to 60 min. This observation was in accordance to results by Jahn and Nielsen (1995), Frølund *et al.* (1996), as well as Wu and Xi (2009) who described this method as gentle to biofilm cells, based on G6PDH activity measurements. Also application of other markers as indicators of cell lysis, such as increased DNA (Azeredo *et al.*, 1999; Comte *et al.*, 2006) or ATP (Azeredo *et al.*, 2003) content in the EPS, as well as live/dead staining (Wu and Xi, 2009) showed no damage to cells in diverse biofilms caused by EPS isolation by CER. However, prolonged duration of the CER isolation may result in stress on the microorganisms, reflected by the slight decreasing trend of culturability of biofilm cells, as occured in the present study, as well as indicated by Jahn and Nielsen (1995). This may be caused by the removal of cations such as Mg^{2+} from the outer membrane by CER, causing its destabilization (Jahn and Nielsen, 1995), or due to the higher shear stress, causing physical damage to the cells (Taylor Eighmy and Bishop, 1985; Jahn and Nielsen, 1995). Shaking in presence of CER for 20 min was found to be optimal for the isolation of EPS from drinking-water biofilms, showing significantly increased yields of EPS components compared to the control treatment by shaking and no noticeable detrimental impact on cells.

Compared to the control EPS isolation by shaking, CER isolation showed highest increase in yields for DNA, followed by proteins, while increase in polysaccharide yields was lowest. This can be attributed to the polyanionic character and higher charge density of the polymers, in particular DNA, or acidic proteins, which allow for cross-linkages of the polymers with the aid of multivalent cations, and consequently, firm binding of the polymers within the EPS matrix. Polysaccharides in drinking-water biofilms, on the other hand, were shown to be composed mainly of non uronic acid-containing carbohydrates, offering lower amounts of binding sites for multivalent cations, and being to a higher degree bound in the EPS matrix by forces other than electrostatic bridging. Hence, removal of multivalent cations by CER isolation could have affected DNA and proteins to a higher degree, compared to polysaccharides. Similar findings have been shown by Higgins and Novak (1997), who demonstrated that proteins readily bind to divalent cations leading to their precipitation. Once divalent cations have been displaced in their study by the addition of monovalent ions, solubility of proteins increased. Also Park and Novak (2007) described higher isolation yields of proteins compared to polysaccharides from activated sludge. Increases in extraction yields of

carbohydrates are thought to be caused mainly mechanically, due to the higher shear imposed by shaking with the CER compared to simple shaking. Alternatively, polysaccharides, which were bound directly to proteins, such as lectins or lectin-like proteins, potentially present in drinking-water biofilms, could have been liberated due to the release of the proteins (Park and Novak, 2007).

The higher isolation efficiency of CER isolation compared to simple shaking treatment can be attributed to the removal of multivalent cations from the EPS, in particular Ca^{2+} or Mg^{2+}. These ions are characteristic components of drinking water and were accumulated from the water phase in elevated concentrations within drinking-water biofilms cultivated in this study. 20 min CER isolation was sufficient for an almost complete removal of Ca (> 86 %) and Mg (> 56 %) from the biofilm. To some extent Fe (37 %) and Cu (44 %) could be removed from the biofilm. The mobilization of Fe is contrary to results by Park and Novak (2007), who demonstrated no effect of CER treatment on Fe or Al concentrations in activated sludge after CER treatment. Wilén *et al.* (2003) found strong correlations between concentrations of total EPS isolated by CER and concentrations of Ca^{2+} and Mg^{2+}, while the correlations with Fe^{3+} or Al^{3+} were low, and postulated, that the general mechanism of EPS matrix disintegration by CER was mainly due to the removal of divalent cations, and not trivalent cations (Wilén *et al.*, 2003; Park and Novak, 2007). However, Fe can exist as trivalent Fe(III), as well as divalent Fe(II). The divalent form was not considered in the study by Wilén *et al.* (2003), as it usually constitutes a low proportion of total Fe in activated sludge (Nielsen *et al.*, 1997). The removal of Fe from drinking-water biofilms in the present study may indicate presence of Fe(II) bound in the EPS of drinking-water biofilms, which, unlike the oxidized Fe(III), could interact with the CER due to its divalent character. Though Fe in drinking water exists solely as Fe(III), iron-reducing bacteria, which have been shown to reside in drinking-water biofilms (Teng *et al.*, 2008; Wang *et al.*, 2012), may have utilized Fe(III), which was accumulated in the biofilm matrix as electron-acceptor, reducing it to Fe(II).

Despite the relatively low removal rates of Fe and Cu from the biofilm suspension by CER treatment, final EPS solutions contained only 6.8 % and 4.1 % of the initial concentration of these cations, respectively. This indicates that Fe and Cu were additionally removed in the course of EPS clean-up by centrifugation and filter sterilization. This can have various explanations. Similarly to Fe, also Cu exists in two oxidation states, Cu(I) and Cu(II), both of

which are stable in water (Mikolay *et al.*, 2010). Both, the reduced Cu(I) as well as the oxidized Fe(III) could have remained attached to the cell fraction, due to a potentially low affinity of the CER towards the cations in these oxidations states. Moreover, irrespective of their oxidation state, Fe and Cu could have been firmly bound to an EPS fraction, which could not be isolated by CER treatment, to cellular material, or present within the cells. Alternatively, Fe and Cu could be present in a particulate and insoluble form, which could explain the particulate matter found during SEM analysis (Fig. 4.5), and which would sediment during centrifugation.

- *Formaldehyde/NaOH* -

Formaldehyde/NaOH has been mainly applied for the isolation of EPS from activated sludge (Liu and Fang, 2002; Comte *et al.*, 2006a). Formaldehyde is believed to "fix" cells by chemically reacting with amino, hydroxyl, carbonyl and sulfhydryl groups of proteins and nucleic acids in the cell membrane, to prevent lysis during EPS isolation. NaOH on the other hand strongly increases the pH, causing dissociation of acidic functional groups of EPS and repulsion of negatively charged EPS components (Liu and Fang, 2002; Comte *et al.*, 2006a,b; D'Abzac *et al.*, 2010).

Treatment of drinking-water biofilms with formaldehyde/NaOH resulted in interference of the chemicals with analysis of EPS components as well as a complete loss of culturability. The loss of culturability may indicate impairment of cells or, in the worst case, even potential cell lysis, causing a contamination of the EPS with intracellular material. This observation is in agreement with Wu and Xi (2009), who indicated cell lysis induced by NaOH treatment applied for EPS isolation from pure culture biofilms of *Acinetobacter* sp. strain AC811 using live/dead staining. Increased DNA content in the EPS has often been used as an indication for cell lysis due to EPS isolation procedures, and has also been applied in various studies to detect cell lysis due to formaldehyde, NaOH or combinations of formaldehyde with NaOH (Tab. 1.2). The DNA yield of formaldehyde/NaOH isolation from drinking-water biofilms was, with exception of the control method by shaking, lowest, which would be contrary to the assumption of cell lysis. However, the high pH of the EPS solution of pH > 14 after addition of NaOH may have resulted in a decreased fluorescence yield of the PicoGreen dye applied for

DNA quantification in this study. According to the manufacturer, PicoGreen is effective in a pH range of 7 to 8, with an optimum pH of 7.5 (Molecular Probes, 2008). An elevated pH during DNA measurement in the current study could have inhibited correct binding of the dye. Furthremore, PicoGreen mainly binds to double-stranded DNA, while contributions of single-stranded DNA or RNA to the signal intensity are negligible (Singer *et al.*, 1997; Molecular Probes, 2008). DNA in the EPS would be denatured and consequently single stranded at a pH > 14. Also hydrolysis of DNA at such a high pH value needs to be considered. Dialyzed EPS solutions usually showed higher DNA contents, which may be attributed to a decrease of pH during dialysis to pH 9.5, allowing for enhanced binding of the PicoGreen reagent to DNA. Alternatively, the decrease in pH value may have allowed for a partial reformation of hydrogen bonds and reannealing of the DNA strands and, consequently, an improved binding of the PicoGreen reagent. Therefore, DNA concentrations determined after EPS isolation by formaldehyde/NaOH are very likely to be underestimated.

Formaldehyde/NaOH isolation, furthermore, caused an interference with the phenol/sulphuric acid determination of carbohydrates, showing much higher concentrations than were determined for the total biofilm. Formaldehyde was identified as the main interference-causing agent in this study, resulting in yellow discoloration in deionized water in the phenol/sulphuric acid assay, despite absence of carbohydrates. Comte *et al.* (2006a) also suspected formaldehyde to contaminate their EPS samples isolated from activated sludge and to chemically react with the polymers, modifying their properties and producing an altered infrared spectrum when EPS isolation involved formaldehyde treatment compared to EPS isolated by alternative methods.

Also the protein determination of EPS isolated with formaldehyde/NaOH was most likely underestimated, which was attributed to both, formaldehyde as well as NaOH. The interference in both assays could, for the most part, be reduced by dialysis. Hence, elimination of formaldehyde and NaOH from the EPS solution by dialysis is essential for this type of EPS isolation. Analysis of EPS proteins isolated by formaldehyde/NaOH by means of 2DE showed a smear of proteins in the acidic part of the gel. The high pH value most likely denatured, or to some extent hydrolysed proteins. The denaturing effect of NaOH on proteins has also been suspected by Karapanagiotis *et al.* (1989), who applied gel-

permeation chromatography for the analysis of EPS isolated from digested sewage sludge by NaOH treatment. The molecular size distribution of EPS isolated by NaOH in their study showed a much higher heterogeneity compared to EPS isolated by other methods (Karapanagiotis *et al.*, 1989). Consequently, treatment of biofilm suspensions with formaldehyde/NaOH can alter the pI and the molecular weights of proteins and peptides and most likely caused the smear of proteins observed on 2DE gels in the present study.

Application of formaldehyde and NaOH separately as well as in combination is, therefore, considered inadequate for EPS isolation from drinking-water biofilms, as well as for the applied quantitative and qualitative analyses.

- *EDTA* -

EDTA has been applied for EPS isolation from a variety of biofilms (Tab. 1.2). EDTA chelates multivalent cations which cross-link EPS polymers, resulting in the destabilization of the EPS matrix and enhanced solubilization of EPS components. Unlike CER, which is separated from the biofilm suspension by sedimentation in the course of EPS clean-up, EDTA remains in the EPS solution and requires additional measures for removal.

The use of EDTA showed negative effects on culturability of drinking-water biofilm cells as well as subsequent analyses. The culturability of biofilm cells decreased in the present study by two orders of magnitude, which may indicate stress imposed on cells. Chelation of cations by EDTA has been shown to cause disordering of the cell membrane of some microorganisms, dissociating proteins and phospholipids, and releasing LPS from the outer membrane of Gram-negative bacteria (Matsushita *et al.*, 1978; Knirel *et al.*, 1990). Therefore, cell lysis during EPS isolation from drinking-water biofilms by EDTA was likely promoted. Interference of EDTA with the Lowry assay has been documented by several authors, including Neurath (1966) or Peterson (1979). They described an increase in colour intensity due to reduction of the phosphomolybdate in the Folin-Ciocalteu's phenol reagent caused by EDTA. In the present study, as soon as the Folin-Ciocalteu's phenol reagent was added, a precipitate formed, which caused a strong increase in absorbance. Dialysis of EPS solutions was applied to remove EDTA and other low molecular weight substances from the samples. Results of the present study indicated that dialysis was insufficient to entirely

eliminate EDTA from the samples, as protein determination in the EPS solutions showed significantly higher concentrations compared to those determined for the biofilm suspensions. EDTA can form complexes with EPS polymers (Liu and Fang, 2002; D'Abzac *et al.*, 2010). Therefore, it is likely that EDTA remained in the sample, and continued to cause interference even after sample clean-up by dialysis. Dialysis of EPS solutions containing EDTA had a further detrimental effect. A 2 fold increase in sample volume and, therefore, a likewise dilution of the EPS solution was observed during dialysis. Such dilution of samples can cause inaccuracy in measurements, especially in case of protein concentrations close to the limit of detection. Neurath (1966) showed that addition of $CaCl_2$ in equimolar concentrations to EDTA to the sample, for the most part, eliminates the interference caused by EDTA. Addition of $CaCl_2$ results in formation of CaEDTA complexes, and thus reduces the detrimental effect of EDTA. This effect was confirmed in the present study, using BSA solutions as reference (results not shown). However, additional manipulation and dilution of the sample by addition of $CaCl_2$ solution decreases the accuracy of subsequent measurements. Analysis of EPS-proteins isolated by EDTA by 2DE proved to be impossible without additional clean-up steps. EDTA itself in concentrations as applied in this study (1 % w/v) or EDTA-EPS complexes could have clogged the pores of the IEF gel. Alternatively, DNA, which is known to interfere with IEF by increasing the viscosity of the sample and by clogging the pores of the IEF gel, could have been insufficiently digested during EPS clean-up, due to the presence of EDTA. EDTA is commonly used for the inactivation of nucleases by chelating Mg^{2+} ions necessary for the functionality of these enzymes. Therefore, it is possible that the DNA present in the EDTA-isolated EPS was not or only partially digested and caused the interferences during IEF.

- *Heat* -

Heat treatment of biofilm samples at 70 °C to isolate EPS yielded the highest concentrations of isolated polymers without any apparent disturbances on the protein, carbohydrate and DNA assays or 2DE. However, culturability of biofilm cells decreased below the limit of detection. Therefore, it is assumed that cell lysis occurred, which contaminated the EPS with intracellular material, leading to increased polymer concentrations within the EPS. Cell lysis by heat treatment was also suspected in studies by D'Abzac *et al.* (2010), who applied heat

treatment at 80 °C for EPS isolation from activated sludge. Furthermore, denaturation of EPS components at a temperature of 70 °C is likely to occur (Karapanagiotis *et al.*, 1989; Comte *et al.*, 2006a; D'Abzac *et al.*, 2010). Hence, biochemical characterization of polymers, in particular enzymes, can be inhibited.

- *Cell lysis* -

Cell lysis induced by the EPS isolation procedure has to be kept at a minimum, as increased lysis rates result in higher amounts of intracellular polymers than would be present in the native biofilm, and therefore, would lead to misinterpretations of EPS analysis. Cell lysis induced by the EPS isolation methods has often been disregarded in literature (Tab. 1.2), probably due to the difficulty of its detection. Nevertheless, cell lysis caused by EPS isolation treatment needs to be minimized, if not completely excluded, to be able to reliably evaluate efficiency of EPS isolation methods. Common indicators for cell lysis applied in studies included increased DNA or protein content (Brown and Lester, 1980), KDO (Platt *et al.*, 1985), activity of the strictly intracellular enzyme G6PDH (Platt *et al.*, 1985), protein/polysaccharide ratio (Karapanagiotis *et al.*, 1989), culturability (Jahn and Nielsen, 1995) or membrane integrity (Wu and Xi, 2009). However, each of these methods has weaknesses. Increased DNA or protein contents, or protein/polysaccharide ratios caused by the EPS isolation method are unsatisfactory markers for cell lysis, as these polymers constitute the EPS matrix and have been shown to be released in varying amounts, based on the different selectivity of EPS isolation methods towards EPS polymers (Park and Novak, 2007). KDO is a constituent of lipopolysaccharides bound to the outer membrane of Gram-negative bacteria (Karkhanis *et al.*, 1978). Increased presence of KDO after EPS isolation indicates shedding of the outer membrane due to the isolation procedure (Matsushita *et al.*, 1978), however, cells do not necessarily have to be lysed and could recover from the isolation treatment. Furthermore, KDO needs to be present in sufficient concentrations to be able to detect outer membrane damage. This was not the case in the current study for drinking-water biofilm cells. G6PDH are strictly intracellular enzymes involved in glucose metabolism (Ng and Dawes, 1973), therefore, increased activity of G6PDH in the EPS after isolation treatment would indicate cell lysis. Presence of G6PDH within drinking-water biofilm cells was confirmed in this study. However, G6PDH activity in the EPS isolated by

shaking or CER treatment was not detected, indicating no cell lysis induced by both methods. Determination of G6PDH activity in EPS isolated by formaldehyde/NaOH, EDTA or heat was not attempted. The reason was, the high pH value during NaOH or EDTA isolation, as well as prolonged exposure to high temperatures, as was the case during heat isolation, can denature enzymes, and thus, inactivate them. EDTA can, furthermore, complex cations, such as Mg^{2+}, necessary for the functionality of the enzymes, decreasing their activity. Hence, activity of G6PDH cannot be applied as reliable indicator of cell lysis for these isolation methods. Culturability of cells after EPS isolation treatment can be applied as measure to assess viability of cells after EPS isolation procedures (Jahn and Nielsen, 1995; Sesay *et al.*, 2006). Decrease of culturability does not necessarily indicate cell lysis, as cells could for example enter the VBNC state due to the treatment. However, decreased culturability suggests stress on biofilm cells imposed by the EPS isolation method, which in the worst case may result in lysis. The Live/Dead BacLight bacterial viability kit allows for discrimination between cells with intact cell membranes and cells with compromised membranes, based on the penetration of the membrane impermeable propidium iodide into the cell and its displacement of the counterstain Syto 9 (Ben-Amor *et al.*, 2005). However, difficulties in application, in particular for multi-species biofilms, have been described (Lawrence *et al.*, 1996; Barbesti *et al.*, 2000; Hannig *et al.*, 2010). Concentrations of the dyes need to be adjusted to the sample, as propidium iodide applied in too high concentrations may enter cells regardless of an intact membrane (Barbesti *et al.*, 2000; Stocks, 2004; Berney *et al.*, 2007). The appropriate concentrations may vary greatly between species, hence, application for multi-species samples can be problematic. Furthermore, lysed cells, which have released their entire cytoplasmic components, would remain undetected. The use of propidium monoazide has been shown to provide more reliable results for the distinction between live and dead cells compared to propidium iodide (Nocker *et al.*, 2007). This stain, however, has not yet been applied for determination of cell damage caused by EPS isolation methods. The variety of methods for the analysis of cell damage and their limitations reflect the difficulty to reliably detect cell lysis induced by EPS isolation methods. Thus, application of several methods for determination of cell integrity after EPS isolation is suggested.

Table 5.1: Advantages and disadvantages of EPS isolation methods for the analysis of EPS from drinking-water biofilms.

Method for EPS isolation	Advantages	Disadvantages
Shaking	+ no cell lysis + no interference with analytical methods + no use of toxic chemicals	- isolation of soluble EPS only
CER	+ increased EPS isolation yields compared to shaking + short durations for EPS isolation + physical and chemical destabilization of EPS + no cell lysis + easily and completely removed from sample + no interference with analytical methods + no use of toxic chemicals	- potential shedding of outer membrane components
Formaldehyde/NaOH	+ increased EPS isolation yields compared to shaking	- potential cell lysis - interference with analytical methods - inaccuracy of quantification of EPS components - dilution of sample - dialysis is essential - chemical reactions with EPS components - denaturation or hydrolysis of EPS components - time-consuming - use of toxic chemicals
EDTA	+ increased EPS isolation yields compared to shaking	- potential cell lysis - interference with analytical methods - inaccuracy of quantification of EPS components - dilution of sample - poorly removed by dialysis - chemical reactions with EPS components - complexation of metal ions required by certain enzymes - time-consuming
Heat	+ increased EPS isolation yields + no interference with analytical methods + no use of toxic chemicals	- cell lysis - denaturation or hydrolysis of EPS components

Chemicals applied for EPS isolation interfered with EPS analysis as well as culturability of biofilm cells. Despite numerous studies dealing with EPS isolation from various types of biofilms, effects of chemical agents applied for EPS isolation on the biochemistry of the EPS

constituents, as well as interferences with subsequent analytical methods have been considered by only few authors, in particular Comte *et al.*, (2006a,b) and D'Abzac *et al.* (2010). Results of the current study showed that EPS isolation should always be selected and adjusted according to the sample type as well as the objective of a study, considering its effects on the biochemistry of polymers and applied biochemical analyses. The optimized CER isolation proved to be the most suitable procedure for the isolation of EPS from drinking-water biofilms, yielding increased amounts of EPS components without detrimental impact on biofilm cells or subsequent analyses. This method was, therefore, applied for further analysis of EPS from drinking-water biofilms.

5.2 Drinking-water biofilm formation on the elastomer EPDM

Established drinking-water biofilms have been extensively studied in real drinking water distribution systems, and in a low number of studies also in domestic plumbing systems, in terms of total cell count, culturability, or population diversity. However, the composition of the EPS matrix of drinking-water biofilms and the dynamics of EPS components throughout the different stages of biofilm formation has not been addressed so far. In this study the established methods for biofilm cultivation, EPS isolation, and analysis were applied to investigate the progress of drinking-water biofilm formation on EPDM over a period of 28 d in two independent plumbing systems. Twice per week biofilms were sampled and analyzed by microbiological, biochemical and molecular biological methods.

5.2.1 Colonization of EPDM by drinking water microorganisms

The EPDM rubber used in the present study was found to be an adequate material for microbial colonization and development of drinking-water biofilms. The exposure of EPDM coupons in biofilm reactors to a continuous flow-through of drinking water resulted in macroscopically visible biofilms. The main colonization on the elastomeric material occurred within the first week of cultivation. Within 11 to 14 d of cultivation biofilm growth reached a quasi-stationary state in which the total cell number remained relatively constant between 1 x 10^8 and 3.3 x 10^8 cells cm^{-2} at plumbing system A, or between 4.1 x 10^8 cells cm^{-2} and

5.1 x 10^8 cells cm^{-2} at plumbing system C. This state may be attributed to the shear imposed by the drinking-water flow, or grazing protozoa controlling cell densities in the biofilms (Pedersen, 1990; Kalmbach *et al.*, 1997). Culturability of drinking-water biofilm cells was relatively high and ranged between 7.4 % to 43.6 % of the total cell count. Cell density and proportion of culturable microorganisms revealed similar orders of magnitude to those determined by Kilb *et al.* (2003) in biofilms grown on EPDM-coated valves in real drinking water distribution systems, but slightly lower values compared to results by Bressler *et al.* (2009), who used a similar experimental system and type of EPDM for the cultivation of drinking-water biofilms as applied in the current study. In contrast to an EPDM, which meets microbiological recommendations according to DVGW Code of Practice W270, and which was applied by Moritz *et al.* (2010) in a similar experimental system, one order of magnitude higher total cell counts were obtained in the present study in 14 d-old drinking-water biofilms. Compared to findings by Wingender and Flemming (2004), who investigated the colonization of steel, copper, polyethylene an polyvinyl chloride coupons exposed to drinking water over a period of 12 to 18 months, total cell counts in the current study were 1 to 2 orders of magnitude higher, while culturability was 2 to 3 orders of magnitude higher. The differences were even more evident when comparing the results obtained in this study to those determined for 2 to 99 year old pipe sections from various German drinking-water networks with established biofilms. These pipe sections revealed total cell numbers between 4 x 10^5 and 2 x 10^8 cells cm^{-2} and an HPC in the range of 10^1 to 2 x 10^5 cfu cm^{-2} (Wingender and Flemming, 2004).

Comparative studies of different types of plumbing materials showed, that elastomeric materials such as EPDM always resulted in the most extensive development of biofilms with relatively high proportions of culturable cells (Rogers *et al.*, 1994; Moritz *et al.*, 2010; Waines *et al.*, 2011). The high accumulation of cells found in this study and in afore mentioned comparative studies is an indication for a high availability of nutrients within the biofilms, which can be attributed to leaching of biodegradable substances from the EPDM rubber material. Rogers *et al.* (1994) demonstrated that elastomeric materials such as latex or ethylene-propylene tend to release considerable amounts of organic carbon into the water phase when exposed to water, which can support excessive biofilm growth. Typical formulations of EPDM rubbers include filler, pigments, plasticizer, curing agents and

additives. If these substances are biodegradable (e.g. plasticizers such as phthalates) biofilm growth may be promoted (Rogers *et al.*, 1994). Another indication for a rather elevated organic load is the high ratio of HPC to total cell counts. A low proportion of HPC in relation to total cell count is typical for drinking water distribution systems and was shown to range between 0.0004 % to 3.5 % on 2 to 99 year old pipes (Wingender and Flemming, 2004). The current study showed significantly higher proportions between 7.4 % and 43.6 %, which was similar to findings by Kilb *et al.* (2003) for biofilms on EPDM rubber-coated valves, as well as Bressler *et al.* (2009) for biofilms on the same EPDM material as applied in the current study, and Moritz *et al.* (2010) for biofilm on EPDM meeting microbiological requirements for use in the drinking water sector.

5.2.2 Dynamics of population diversity of drinking-water biofilms

Population diversity of drinking-water biofilms grown on EPDM was analyzed by DGGE of PCR-amplified 16S rDNA fragments and monitored throughout cultivation periods of up to 28 d. DGGE analysis revealed a high diversity of microorganisms present in drinking-water biofilms, showing a maximum of 66 bands for biofilms cultivated at plumbing system A, and 45 bands for biofilms cultivated at plumbing system C, each band ideally representing one bacterial species. Similar diversity was obtained by Bressler *et al.* (2009), detecting 68 bands in drinking-water biofilms cultivated for 14 d on a similar formulation of EPDM and experimental setup. Initial colonization of the EPDM coupons was shown to be attributed to a small number of organisms, the bands of which were detected in the biofilms in varying intensities throughout the experimental run. The most significant increase in microbial diversity occurred within the first 7 d and from then on remained relatively uniform throughout the experimental run, which was reflected by a high similarity of 70 % to 93 %. The dynamics of population diversity in drinking-water biofilms has been investigated only in a small number of studies in literature. Similar results to those described in the current study have been presented by Deines *et al.* (2010) for drinking-water biofilms cultivated for up to 11 d on polyethylene in a pilot-scale drinking water distribution system. Deines *et al.* (2010) detected one species colonizing the surface of their model distribution system immediately after its exposure to drinking water, which persisted in the biofilms throughout the

experimental period. In their study the most significant increase in population diversity occurred within the first 3 days. From then on population diversity remained relatively constant showing similarities of DGGE band patterns of > 70 %, indicating low variation of microbial composition of drinking-water biofilms with time. However, the similarity coefficients calculated in the study by Deines *et al.* (2010), as well as in the present study only consider presence or absence of bands, while variations of band intensity are not regarded. Slight variations of band intensities of a number of bands were detected in both studies in the progress of biofilm growth, demonstrating minor, but noticeable changes of composition of drinking-water biofilm populations. Lee *et al.* (2005) on the other hand found relatively low similarity of DGGE band patterns, of drinking-water biofilms cultivated on galvanized iron wafer in a model drinking-water distribution system connected to a laboratory tap. However, their DGGE profiles indicated relatively low population diversity, showing a maximum of 11 bands. This may be attributed to the lower amount of PCR product (approximately 200 ng) applied on the gels compared to the amount applied in the present study (500 ng), or to the staining by ethidium bromide, which is generally less sensitive compared to SybrGold applied here. Considerable changes in the composition of bacterial biofilm populations have been shown in studies performed by Kalmbach *et al.* (1997) on drinking-water biofilms cultivated on glass or polyethylene slides in a Robbins device connected to domestic water distribution systems, using α-, ß- or γ-Proteobacteria specific FISH probes. Their analysis of the distribution of the different classes of Proteobacteria displayed fluctuations of abundance as well as dominance of certain groups with increasing drinking-water biofilm age. Results of the current study indicate that during the initial stages of biofilm formation of 5 d to 7 d a population composition is establishing. Established drinking-water biofilms exhibit a stable composition of microbial communities with only minor changes during biofilm maintenance. Hence, considering real drinking water systems, the period directly after installation of materials into a drinking water system and startup of operation is the most dynamic and amenable in respect to population diversity during the formation of drinking-water biofilms.

5.2.3 Biochemical composition of drinking-water biofilms

The biochemical composition of drinking-water biofilms and their EPS and the progress of polymer formation throughout cultivation periods of up to 28 d at plumbing system A and plumbing system C were quantitatively and qualitatively analyzed. Despite the misconception in early biofilm studies that polysaccharides make up the entire extracellular matrix, more recent studies, mostly dealing with activated sludge, have identified proteins as major components of the EPS (Jahn and Nielsen, 1995; Frølund *et al.*, 1996). The present study shows that proteins are the dominant polymers within the total biofilm as well as the EPS matrix, which was irrespective of biofilm age, followed by polysaccharides. eDNA, which is considered to have structural functions within the biofilm matrix (Whitchurch *et al.*, 2002; Böckelmann *et al.*, 2006), was also present in considerable amounts in the EPS of drinking-water biofilms. The overall protein content calculated per surface area increased continuously throughout the cultivation period in biofilms cultivated at plumbing system A as well as plumbing system C. However, protein amounts considered per cell showed an altered development. During the initial stages of biofilm formation protein contents decreased until day 14 to 21 of cultivation. Once biofilms reached 14 d to 21 d of age protein contents per cell increased again until the end of the experimental run. Carbohydrates demonstrated different developments, depending on the drinking water source. Carbohydrates of drinking-water biofilms cultivated at plumbing system A revealed a similar progress throughout the cultivation period as was described for proteins. On the other hand, carbohydrate concentrations of drinking-water biofilms cultivated at plumbing system C remained constant once the biofilms reached 14 d of age. Furthermore, carbohydrate concentrations from plumbing system C biofilms calculated per cell showed no further increase, unlike protein concentrations per cell in biofilms > 14 d of age. DNA concentrations also showed constant concentrations once biofilms reached 11 d (plumbing system C) or 21 d (plumbing system A) of age. This may either indicate a production stop of carbohydrates and eDNA, once a mature biofilm was established, or an equilibrium between production of carbohydrates and eDNA, and their degradation by hydrolytic enzymes, expressed in higher quantities with increasing biofilm age. Furthermore, Whitchurch et al. (2002) demonstrated, that eDNA is of major importance during the initial stages of *P. aeruginosa* biofilm formation. The high eDNA concentrations per cell in the EPS during the

initial stages of biofilm formation in the present study may also imply such a profound role of eDNA for young biofilms. However, the role of eDNA requires further investigations.

Analysis of extracellular proteins in this study was performed by use of 2DE in order to qualitatively analyze changes in the extracellular protein diversity throughout the cultivation of drinking-water biofilms. Similarly to the increase in overall EPS protein concentration throughout the cultivation run, also the diversity of EPS proteins increased for the initial 11 d to 14 d. Further cultivation of biofilms, however, resulted in a significant decrease of the diversity of proteins. The amount of proteins with high molecular weights, as well as those with high pI disappeared with increasing biofilm age. This may indicate degradation and turnover of proteins by proteases *in situ* or in the course of EPS isolation and clean-up. To exclude degradation of proteins in the course of EPS isolation, a protease inhibitor cocktail was added to drinking-water biofilms cultivated at plumbing system C immediately after harvesting of biofilms from the coupons. This cocktail was chosen as it inhibits the action of a variety of proteolytic enzymes, including serine, cysteine, aspartic and metalloproteases, as well as aminopeptidases. However, this phenomenon was still observed after addition of the protease inhibitor cocktail, indicating increased production of proteases once biofilms reached an age of 14 d, degrading proteins within the established biofilm.

Increased production of proteases in drinking-water biofilms > 11 d of age was confirmed by gel electrophoresis of total biofilm as well as EPS samples on zymogram gels. The gels contained gelatin as substrate, which was not degraded during the initial stages of biofilm formation. However, once biofilms reached 11 d of age, activity bands in the zymogram gels appeared, indicating gelatin degradation by proteases. The diversity of activity bands on the zymogram gels increased with increasing biofilm age, showing a maximum of 13 distinct bands for the total biofilm sample and 9 bands in the EPS after 28 d of cultivation.

To demonstrate and quantify expression of cell-associated as well as extracellular enzymes during the formation of drinking-water biofilms, activities of seven further groups of enzymes were analyzed. A microtitre plate assay was designed in the present study to be able to analyze all seven enzymes groups at once (Fig. 3.4). The enzyme groups included peptidases, α-D-glucosidases, ß-D-glucosidases, N-acetyl-ß-D-glucosaminidases, lipases,

esterases, and phosphatases, all of which belong to the class of hydrolases (Wingender and Jaeger, 2002). These groups represent enzymes commonly detected in naturally occurring biofilms, as well as biofilms in technical systems (Tab. 1.1), and are involved in the degradation of macromolecules, such as polysaccharides, proteins, DNA or lipids, into low molecular weight substances. Activities of all enzyme groups were detected in all fractions of drinking-water biofilms, including the total biofilm, biofilm cells after EPS isolation, as well as in the EPS. The following order in turnover rates was determined: esterases > lipases > phosphatases ≥ N-acetyl-ß-D-glucosaminidases > peptidases ≥ α-/ß-glucosidases. A similar hierarchy was determined by Emtiazi *et al.* (2004) for esterases, peptidases and glucosidases in drinking-water biofilms cultivated at different location in a drinking water distribution system. Results of their study showed approximately 10 fold higher esterase activity than peptidase activity in their biofilms (Emtiazi *et al.*, 2004). In the present study the difference between these two enzyme groups was even more pronounced, showing a three orders of magnitude higher esterase activity than peptidase activity. High enzymatic activities can indicate a high availability of substrates (Emtiazi *et al.*, 2004). Naturally occurring substrates for esterases are for example lipidic compounds, such as short-chain fatty acids. The significantly higher difference between esterase and peptidase activity in this study compared to results by Emtiazi *et al.* (2004), however, may indicate an additional source for esterases substrates, such as compounds leaching from the substratum used for biofilm cultivation. In contrast to the stainless steel substratum applied by Emtiazi *et al.* (2004), the EPDM material used in the current study is known to release considerable amounts of TOC. This can for example include plasticizers, which are applied as additives for elastomers like EPDM to alter physical and mechanical properties of the material (Webb *et al.*, 2000). Commonly applied plasticizers for elastomeric materials in the drinking water sector belong to the class of organic esters, in particular bis(2-ethylhexyl) adipate, which is an ester of diethylhexanol and adipidic acid, or phthalates, which are composed of phthalic acid and saturated C-8 (octyl) to C-10 (decyl) alcohols (Umweltbundesamt, 2011). Due to their structure, organic ester-based plasticizers represent potential substrates for esterases, and have been shown to be degraded by extracellular esterases produced by bacteria or fungi (Pantke, 1996; Flemming, 1998; Webb *et al.*, 2000). Hence, the high esterase activity in this study may be attributed to the high abundance of organic ester-based plasticizers, leached

from the EPDM and accumulated in the EPS matrix, which can be extracellularly degraded to aid in their utilization as carbon sources.

Such a pronounced activity in comparison to activities of other enzyme groups has also been described for different environmental biofilms other than drinking-water biofilms, for example activated sludge (Frølund *et al.*, 1995), littoral sediments (Boschker and Cappenbert, 1998) and soil (Vepsäläinen *et al.*, 2004). However, susceptibility of the esterase substrate MUF-butyrate applied in this study to enzymatic hydrolysis has been demonstrated for enzymes other than esterases (Krisch, 1971; Jones and Lock, 1989). Though contributions of enzymes other than esterases are generally low (Krisch, 1971), Jones and Lock (1989) proposed that degradation of MUF-butyrate should be considered an estimate of a general hydrolytic activity.

Lipases, which catalyze the cleavage of fatty acids, exhibited second highest activities of all fractions of drinking-water biofilms and indicate high potential for lipid turnover. Activities of phosphatases, which remove phosphate groups from phosphorylated organic substances, and N-acetyl-ß-D-glucosaminidases, which are involved in decomposition of peptidoglycan and chitin, were significantly lower compared to esterase and lipase activity. Peptidases, which break up peptide bonds, and α- or ß-D-glucosidases, which cleave glycosidic bonds of oligosaccharides, were lowest, demonstrating relatively low protein or carbohydrate turnover. The presence of hydrolytic activities of the different groups of enzymes in this study indicated the potential of drinking-water biofilms to degrade wide a variety of polymers.

The progress of enzymatic activity throughout biofilm cultivation of up to 28 d varied between the enzyme groups. Lipases, esterases and phosphatases exhibited a rather continuous increase of activity throughout the cultivation period. Peptidases, α-D-glucosidases, ß-D-glucosidases and N-acetyl-ß-D-glucosaminidases on the other hand, revealed an initial “lag phase” for 11 d to 18 d of biofilm age, followed by an “exponential phase” until the end of the experimental run. This progression becomes even more apparent considering the specific activities of the EPS fraction, indicating a more pronounced increase in the production of extracellular matrix enzymes compared to cell-associated enzymes. A similar progress of total biofilm enzymatic activity was described by Riemann *et al.* (2000) for

leucine-aminopeptidase and ß-glucosidase activity of seawater microorganisms during an algal bloom cultivated for up to 15 d. Lehman and O'Connell (2002) described an "exponential" increase of extracellular ß-glucosidase activity for free-living and attached groundwater organisms after 20 to 40 hours of perfusion of sand-packed columns with groundwater. Romani *et al.* (2008) determined dynamics of leucine-aminopeptidase, ß-glucosidase, and ß-glucosaminidase activities in freshwater biofilms cultivated for up to 50 d, as well as in EPS isolated from these biofilms by CER. Their study revealed the most significant increase in total enzymatic activity once the biofilm growth reached a quasi-stationary state of biomass after approximately 30 d of biofilm age. However, unlike the "exponential" increase of activity in the EPS presented in the current study, extracellular enzymatic activity measurements in their study revealed constant activities throughout the cultivation period (Romani *et al.*, 2008). The increase of peptidase, α-D-glucosidase, ß-D-glucosidase and N-acetyl-ß-D-glucosaminidase activities once the biofilms reached 11 d to 18 d of age in this study coincided with the increase in abundance of proteases, stagnation of carbohydrate and DNA concentrations, increase of total protein quantities per cell, decrease of diversity of proteins on 2D gels, and the quasi-stationary state of total cell numbers. This may indicate changes in gene expression in the course of biofilm maturation towards production of biofilm specific proteins once a mature biofilm has formed. Such changes were for example described by Sauer and Camper (2001) and Sauer *et al.* (2002) for *P. putida* and *P. aeruginosa* biofilms, respectively, investigating production of intracellular proteins of planktonic cells and biofilms at different stages of maturation. Both studies demonstrated global changes in gene expression of biofilm cells compared to their planktonic counterparts during the formation of biofilms, in respect to motility, polysaccharide production and quorum sensing (Sauer and Camper, 2001; Sauer *et al.*, 2002). In the current study modifications in gene expression may be reflected in the extracellular protein concentrations, in particular the concentrations of hydrolytic enzymes, which indicate an expansion of the degradation potential and substrate utilization, and potential changes in the metabolism of biofilm cells. Metabolism during the initial stages of drinking-water biofilm formation could be based on the preferential consumption of products leached from the EPDM substratum. Once a mature biofilm has formed changes in gene expression towards biofilm specific metabolism could have occurred, resulting in the

establishment of an extracellular digestive system of enzymes specific for the turnover of biomass, in particular the metabolism of proteins, carbohydrates or cellular components. The results of this study, therefore, indicate the EPS matrix' metabolic potential and signify the importance of the EPS matrix as extracellular digestive system, predigesting various substrates into low molecular weight matter, which in turn can be readily utilized by biofilm organisms.

5.3 Influence of upstream plumbing materials on drinking-water biofilms

Characteristics of drinking water have substantial influence on the development of a drinking-water biofilm. Besides temperature and pH, which are in general major factors determining biofilm growth, the chemical composition of drinking water has a direct impact on microorganisms in the planktonic or sessile state. The composition may vary depending on the materials implemented in a drinking water installation, due to the deterioration of the material when exposed to drinking water and release of components, such as metal ions in case of metallic materials or organic substances in case of plastic materials, into the water phase. The deterioration can be facilitated by biofilms, in terms of microbially influenced corrosion (Emde *et al.*, 1992). This release can substantially alter water composition, resulting in a different drinking water reaching the consumer's tap compared to the drinking water leaving the water works (Pepper *et al.*, 2004; Flemming *et al.*, 2012). Investigations of drinking-water biofilms in literature are mainly focused on biofilm formation in drinking water distribution systems, while those dealing with drinking-water biofilms encountered in domestic plumbing systems are underrepresented (Eboigbodin *et al.*, 2008). Major differences may occur once the water passes the water meter of a public or private building and enters the plumbing system. Higher water temperatures caused by the pipe's passage of heated rooms, or altered hydrodynamic conditions, i.e. points of stagnation, can be encountered in plumbing systems. Also, the materials used for plumbing systems can differ from those used in drinking water distribution systems (Flemming *et al.*, 2012). Copper is the by far most widely applied material in domestic plumbing systems in Germany (Elfström Broo *et al.*, 2001; Kistemann *et al.*, 2010; Fig. 5.1), due to its wide range of applicability, its simple processibility, its resilence and its resistance towards corrosion (German Copper

Institute, 2010). Furthermore, antimicrobial properties against pathogenic and antibiotic resistant bacteria have been attributed to copper (Domek *et al.*, 1984; Landeen *et al.*, 1989; Artz and Killham, 2002; Wilks *et al.*, 2005; Noyce *et al.*, 2006; Elguindi *et al.*, 2011). However, recent studies have demonstrated the susceptibility of copper materials to microbial colonization in drinking water systems (Moritz *et al.*, 2010).

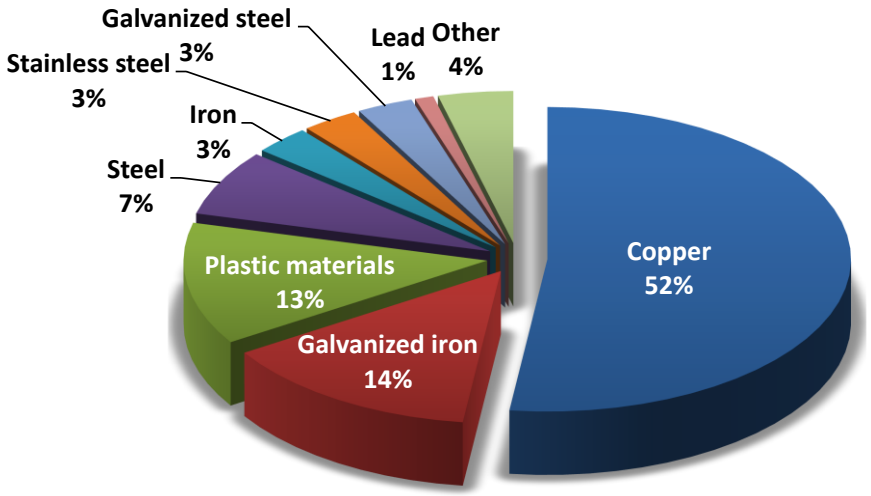

Figure 5.10: Materials used in plumbing systems of public buildings in Germany (according to Kistemann *et al.*, 2010).

In the present study, drinking-water biofilms were grown on EPDM for 14 d in biofilm reactors connected to three plumbing systems made of copper and two distribution systems, which were supplied with drinking water by three different water works. The drinking-water biofilms were microbiologically, molecular biologically and biochemically analyzed and compared. This allowed for determination of the variability of drinking-water biofilms cultivated in different drinking water distribution systems, as well as changes of the biofilm composition caused by plumbing systems made of copper.

5.3.1 Variability of drinking-water biofilms cultivated on EPDM according to water characteristics and plumbing materials

The influence of drinking water characteristics and plumbing systems on drinking-water biofilms cultivated on EPDM and their EPS composition was determined by comparison of

qualitative parameters, including microbial population and extracellular protein diversity, as well as quantitative sum parameters, including total biomass and water content, total cell count, HPC, and protein, carbohydrate and DNA contents after 14 d of biofilm growth.

Drinking-water biofilms grown at the five different locations revealed similar total cell counts between 6.0 x 10^7 cells cm^{-2} and 2.8 x 10^8 cells cm^{-2}, indicating a generally high degree of colonization of the EPDM substratum, irrespective of the water source. Further analyses showed relatively low similarities among the biofilms, in terms of population diversity, culturability or biochemical composition of the biofilms and their EPS. A unique pattern for the community composition of drinking-water biofilms was not observed. Population diversity showed significant differences among drinking-water biofilms according to their origin, which was reflected by similarities of 46 % to 63 %. This indicated pronounced variability of microbial composition within drinking-water biofilms. DGGE band patterns revealed merely three bands out of 26 to 52 in common in all drinking-water biofilms. Such variability has also been described by Emtiazi *et al.* (2004) investigating biofilm community composition of drinking-water biofilms cultivated at two house branch connections within a drinking water distribution system by DGGE and showing similarities of band patterns of 45 % to 56 %. Differences in characteristics of the bulk water, in particular the water temperature and composition of inorganic substances, can strongly influence the microbial composition and formation of drinking-water biofilms (Roeder *et al.*, 2010a). In the present study the most substantial difference was the water temperature, which varied significantly among locations. The water temperature at distribution system A was lowest (11.4 ± 0.9 °C), followed by plumbing system A (17.3 ± 2.9 °C), plumbing system B (19.0 ± 1.9 °C), distribution system B (19.1 ± 2.5 °C) and plumbing system C (22.0 ± 0.7 °C). Studies by Roeder *et al.* (2010a) on drinking-water biofilms cultivated at 12 °C and 37 °C on diverse plumbing materials including EPDM indicated significant changes of community composition caused by different water temperatures. Furthermore, higher similarities of populations cultivated on different materials at similar temperatures were obtained in their study, compared to similarities of populations grown on the same material but different temperatures (Roeder *et al.*, 2010a). Hardness of the waters, which was mainly determined by the Ca concentration, showed significant differences between the water sources, being highest for drinking water from supplier A (Ca: 112 mg L^{-1}), followed by drinking water from

supplier B (Ca: 50 mg L^{-1}), and drinking water from supplier C (Ca: 38 mg L^{-1}). The differences in water temperature and water hardness very likely favored different biofilm flora in the present study. A correlation between water temperature or hardness of the drinking waters and the biochemical composition of drinking-water biofilms and their EPS was not evident.

Proteins, carbohydrates and DNA were detected in all drinking-water biofilms and in isolated EPS. Proteins represented the major component of all drinking-water biofilms and their EPS, followed by carbohydrates and DNA. This finding is in accordance to results by Kilb *et al.* (2003), who quantified proteins and carbohydrates in 5 weeks to 4 years old drinking-water biofilms grown on EPDM coated valves and their EPS isolated by stirring and centrifugation in 10 different drinking water distribution systems. Protein and carbohydrate concentrations in the total biofilm as well as in the EPS fraction in the present study were in the same range as determined by Kilb *et al.* (2003), who showed significant variation among locations of biofilm cultivation (total biofilm protein range: 20.9 to 281.1 µg cm^{-2}; total biofilm carbohydrate range: 6.8 to 131.1 µg cm^{-2}; EPS protein range: 1.2 to 12.9 µg cm^{-2}; EPS carbohydrate range: 0.82 to 6.2 µg cm^{-2}). Variations of the biochemical composition of the biofilms and their EPS in the present study were lower compared to those determined by Kilb *et al.* (2003). Their study, furthermore, showed protein : carbohydrate ratios of up to 6.2 : 1 for the total biofilm and up to 3.4 : 1 for the EPS fraction (Kilb *et al.*, 2003), which were similar to those determined in the present study. Results of the present study as well as of the study by Kilb *et al.* (2003) therefore indicate a strong variability of biochemical composition among drinking-water biofilms. Furthermore, both studies showed that drinking-water biofilms in general represent a type of biofilms, in which proteins constitute the most abundant component, irrespective of biofilm age or the type of drinking water.

All drinking-water biofilms cultivated in the present study exhibited similar characteristics in respect to absorption and accumulation of considerable quantities of metal ions, in particular heavy metals, within the biofilm matrix. The alkaline earth metals Ca and Mg were accumulated to a minor degree within the biofilm compared to heavy metals. Maximally one order of magnitude higher concentration of Ca and Mg were found within the biofilms compared to the water phase. The heavy metals Fe, Cu, Zn, and Al, on the other hand, were enriched by 2 to more than 3 orders of magnitude in the biofilm. Biofilms have been shown to bind and concentrate heavy metals such as Cu, Zn or Cd from dilute aqueous solutions

(White and Gadd, 1998; 2000; Gadd, 2000; 2010). Processes such as ion exchange, chelation or complexation bind heavy metals and thus remove them from the water phase. These processes have been known and exploited for decades in biotechnology, in particular for metal removal in wastewater treatment. In the drinking water sector such processes can cause severe problems, due to the potential precipitation and incrustation of wells or within distribution pipes, obstructing the water flow, clogging pipes and offering microorganisms a protected environment with large and porous surfaces (Emde *et al.*, 1992; Loewenthal *et al.*, 2004)

The plumbing systems in this study, which in all cases were made of Cu, strongly influenced Cu concentrations in the drinking water. More than one order of magnitude higher Cu concentrations were detected in the drinking waters when collected from the plumbing system, compared to the drinking waters taken from the distribution systems, while all other determined inorganic substances remained unchanged. The increased Cu concentration in the water phase in drinking waters from plumbing systems in turn resulted in more than one magnitude higher Cu concentrations in the biofilms compared to those cultivated in the distribution system. Biofilms cultivated at distribution systems on the other hand exhibited higher Fe concentrations compared to their counterparts from plumbing systems. The results of this study indicated changes in biofilm and EPS composition attributed to the increased Cu content in drinking waters from the plumbing systems. Changes in population diversity can be attributed to the plumbing material and accumulation of Cu released from the material in the water phase and within the biofilm matrix, which can favor the growth of certain Cu tolerant microorganisms. Cu in concentrations as detected in drinking water at plumbing system C (0.14 ± 0.04 mg L^{-1}), plumbing system A (0.067 ± 0.019 mg L^{-1}) and plumbing system B (0.037 ± 0.001 mg L^{-1}) can cause inhibitory or toxic effects for certain microorganisms (Thurman and Gerba, 1989; Straub *et al.*, 1995; Artz and Killham, 2002; Kim *et al.*, 2002; Teitzel and Parsek, 2003; Lehtola *et al.*, 2004; Teitzel *et al.*, 2006; Moritz *et al.*, 2010; Dwidjosiswojo *et al.*, 2011). Hence, Cu materials used in plumbing systems can substantially alter the composition of and abundance among microbial populations within a drinking-water biofilm, by increasing Cu concentrations in the drinking water, and thus, inhibiting growth of Cu susceptible microorganisms, while favoring Cu tolerant species.

In the present study, the effect of Cu on drinking-water biofilms was further evidenced by determination of the culturability of biofilm cells on R2A medium. Cu can affect culturability of cells by disturbing metabolic functions, disrupting structure and function of proteins, displacing metal cofactors essential for proteins, or by formation of reactive oxygen species causing oxidative stress or cell damage, or inducing a VBNC state (Teitzel *et al.*, 2006; Moritz *et al.*, 2010; Dwidjosiswojo *et al.*, 2011). Biofilms from plumbing systems contained considerably lower proportions of culturable cells (8.5 % to 15.3 % of the total cell count), compared to the proportions of culturable cells in biofilms from distribution system (46.8 % to 50.5 % of the total cell count). The biofilm wet weight also varied significantly despite similar total cell counts. Plumbing system A and B biofilms exhibited 3.2 fold and 2.1 fold higher biofilm wet weights, respectively, compared to their counterparts cultivated at the respective distribution system. Highest biofilm wet weight was observed for plumbing system C biofilms, which was also exposed to highest Cu concentrations in the drinking water. EPS, in particular hydrophilic polysaccharides and proteins, act hygroscopically attracting and retaining water from the environment (Roberson and Firestone, 1992; Flemming and Wingender, 2010). Exposure to high copper concentrations may have resulted in altered production of EPS with different characteristics in terms of water retention, which may explain higher water contents in plumbing system biofilms exposed to higher Cu contents. An upregulation of EPS production due to Cu stress has been demonstrated by Keith and Bender (1999) by means of increased alginate production in *P. syringae* exposed to elevated Cu concentrations. The yields of isolated EPS, in particular polysaccharides and proteins, varied considerably among biofilms. A decreasing trend of isolated EPS components with increasing Cu concentrations in the drinking waters was observed. Biofilms cultivated at plumbing system C, which contained highest concentrations of Cu, yielded relatively low proportions of extracellular carbohydrates (11.3 %) and proteins (4.5 %) isolated from the biofilm by CER treatment, followed by plumbing system A biofilms (carbohydrates: 18.8 %; proteins: 6.1 %). Biofilms cultivated at plumbing system B or distribution system B revealed relatively similar carbohydrate (33.8 % and 24.4 %, respectively) or protein (18.1 % and 16.0 %, respectively) yields, while recovery of carbohydrates (49.7 %) and proteins (34.6 %) from distribution system A biofilms was highest (Fig. 4.27). The influence of Cu on biofilm cells and EPS production can only be

speculated. The differences in EPS yields could for example indicate decreasing EPS component production with increasing Cu content, due to lower activity of cells, which would be contrary to results presented by Keith and Bender (1999). Alternatively, this may demonstrate production of EPS components tightly bound to biofilm cells, which are specifically produced to protect biofilm cells from Cu stress, and which are not readily isolable by the EPS isolation procedure using CER. A further explanation could be a potential complexation and immobilization of Cu by EPS constituents, rendering the EPS' solubility and susceptibility to isolation by CER.

Analysis of EPS proteins by 2DE in general displayed only a limited number of common proteins in the EPS of the five drinking-water biofilms. However, the analysis identified three clusters of proteins in common only in biofilms cultivated at plumbing systems, and consequently, exposed to higher concentrations of Cu, which were not found in biofilms cultivated at distribution systems. This included a protein with significant homology in MS-spectra to a protein involved in the efflux apparatus of cells, i.e. a RND (resistance-nodulation-cell division) family efflux transporter MFP (membrane fusion protein) subunit. Efflux systems are required by microorganisms for example to cope with elevated and potentially toxic concentrations of metals, by "flushing" the metals out of the cytoplasm and through the cell membrane into the environment (Nies and Silver, 1995). Efflux systems for the removal of Cu from the cytoplasm of cells have been described for various organisms, for example involving the Cu resistence operon (cop) of *P. aeruginosa,* or the plasmid-borne Cu resistance determinant (pco) in *E. coli* (Cooksey, 1994; Nies and Silver, 1995; Silver, 1996; Teitzel and Parsek, 2003). Up-regulation of genes encoding for transport system components under Cu stress has been demonstrated (Teitzel *et al.*, 2006) and could play an important role for the resistance of drinking-water biofilm organisms to the encountered elevated copper concentrations in drinking waters and within drinking-water biofilms affected by Cu plumbing materials. A further protein exhibited significant homology of MS-spectrum to a two component response regulator or a two component LuxR family transcriptional regulator. Transcriptional regulators can activate or silence gene expression. In *P. aeruginosa*, for example, the two component transcriptional regulator AlgB is necessary for high-level production of alginate (Wozniak and Ohman, 1991). In *P. syringae* the response regulator AlgT (σ^{22}) controls the activation of alginate production in response to

heat, osmotic stress or oxidative stress, as well as in response to Cu stress (Keith and Bender, 1999). Such regulators may also be involved in Cu stress specific gene expression in drinking-water biofilm cells cultivated in this study, to counteract detrimental effects of Cu.

These protein spot were not detected in the 2D gels of EPS proteins from drinking-water biofilms exposed to low Cu contents at the distribution system A or B. Presence of these proteins in biofilms grown at high Cu levels, therefore, may indicate the significance of these proteins as stress response to the elevated Cu concentrations and upregulation of certain genes for enhanced production of Cu stress specific cell components and EPS.

5.3.2 Incorporation of hygienically relevant microorganisms into drinking-water biofilms

The potential of drinking-water biofilms to accommodate hygienically relevant microorganisms has been demonstrated in several studies, indicating a potential threat for human health (Szewzyk *et al.*, 2000; Flemming *et al.*, 2002; Bressler *et al.*, 2009; Moritz *et al.* 2010). In the present study, drinking-water biofilms were cultivated at plumbing system C for 14 d on EPDM and exposed to a pure culture of *P. aeruginosa* AdS in order to simulate a potential contamination with a hygienically relevant microorganism. The aim was to assess the influence of *P. aeruginosa* on the microbiological and biochemical composition of drinking-water biofilms and their EPS.

Microbiological requirements for drinking water quality are based on cultivation on diverse media, aiming at the determination of indicator parameters, such as colony counts of heterotrophic microorganisms, or microbiological parameters, such as presence of culturable coliform bacteria, *Escherichia coli*, or enterococci, as well as the facultative pathogens *P. aeruginosa* or *Legionella* spec. within the water phase (TrinkwV, 2011). In the current study, presence of *P. aeruginosa* in drinking-water biofilms before and after the inoculation of the biofilm with the facultative pathogen was analyzed by determining colony formation on the *P. aeruginosa*-selective CN agar medium and by the culture-independent method FISH. The analysis of 14 d-old drinking-water biofilms by plating on CN agar or by FISH both revealed no presence of *P. aeruginosa* in native drinking-water biofilms prior to inoculation. The *P. aeruginosa* inoculum exhibited a culturability of $2.1 \pm 0.7 \times 10^6$ cfu ml^{-1} on

CN agar prior to inoculation. Presence of *P. aeruginosa* in the biofilms after exposure of the biofilms to the *P. aeruginosa* inoculum was not detected culturally on CN agar. This was contrary to results obtained by FISH, which detected significant amounts of FISH-positive *P. aeruginosa* cell counts incorporated into the drinking-water biofilm ($1.2 \times 10^5 \pm 5.9 \times 10^4$ cells cm^{-2} 24 h after inoculation; $2.6 \times 10^5 \pm 2.8 \times 10^5$ cells cm^{-2} 7 d after inoculation). Cultivation methods in general have major weaknesses. Cultivation media are selective for certain microorganisms and, therefore, do not reflect the total population of a microbial community (Amann *et al.*, 1995; Moritz *et al.*, 2010; Wingender, 2011; Wingender and Flemming, 2011). Furthermore, the vast majority of microorganisms cannot be cultivated on standard media, and, more importantly, adverse environmental conditions may induce the transition of microorganisms into the VBNC state (Kalmbach *et al.*, 1997; Oliver, 2005; Wingender, 2011). The FISH oligonucleotide probe applied in this study specifically targets intact 16S rRNA sequences, hence, can be applied to indicate viability of cells. However, presence of complementary rRNA sequences from dead *P. aeruginosa* or those from lysed bacteria retained in the EPS matrix cannot be excluded. Nevertheless, FISH results suggests a successful incorporation of *P. aeruginosa* AdS into drinking-water biofilms. The high discrepancy between culturability and FISH indicates possible transition of *P. aeruginosa* AdS into the VBNC state. These results are in accordance with Moritz *et al.* (2010). Even though in their study a proportion of culturable *P. aeruginosa* cells was observed, it was considerably lower compared to the detected FISH-positive cell counts (Moritz *et al.*, 2010). Similar observations were described for *L. pneumophila* in drinking-water biofilms (Långmark *et al.*, 2005; Lehtola *et al.*, 2007; Gião *et al.*, 2009; Moritz *et al.*, 2010). This effect could be associated with the composition of the drinking water used for drinking-water biofilm growth. Exposure of *P. aeruginosa* AdS to the drinking water used for the cultivation of drinking-water biofilms resulted in a decrease of culturability by 5 orders of magnitude, even when the drinking water was diluted 5 fold in deionized water. The decrease in culturability could, to some extent, be correlated to the Cu concentrations in the drinking water. Incubation of *P. aeruginosa* AdS in Cu solutions prepared in 6 mM phosphate buffer (pH 7.0) with Cu concentrations of up to 4 µM showed unhindered culturability of *P. aeruginosa* for Cu ion concentrations of up to 0.6 µM. Increasing Cu ion concentrations of ≥ 0.8 µM caused a gradual decrease of culturability, resulting in 5 orders of magnitude lower colony counts

when exposed to 4 µM Cu solutions. Dwidjosiswojo *et al.* (2011) described a similar decrease of culturability of *P. aeruginosa* in response to incubation in different concentrations of Cu in deionized water, and a successful resuscitation of *P. aeruginosa* once Cu ions were complexed, and hence, rendered harmless. This indicates the transition of *P. aeruginosa* into the VBNC state as stress response to Cu. The transition into the VBNC state in response to exposure to elevated Cu concentrations has also been described for a variety of Gram-negative bacteria, including *Agrobacterium tumefaciens, Rhizobium leguminosarum* (Alexander *et al.*, 1999), *Xanthomonas campestris* pv. *campestris* (Ghezzi and Steck, 1999), *Ralstonia solanacearum, E. coli* (Grey and Steck, 2001a,b), *Erwinia amylovora* (Ordax *et al.*, 2006) and *Xanthomonas axonopodis* pv. *citri* (Del Campo *et al.*, 2009). 5 fold diluted drinking water, which resulted in a decrease of culturability by 5 orders of magnitude in the present study, however, was equivalent to a Cu concentration of 0.44 µM, at which *P. aeruginosa* should be culturable. This indicates that Cu in drinking water was most likely only one of the causative agents inhibiting culturability of *P. aeruginosa* in this study. Free chlorine was not detected in the drinking water, hence, effects of chlorine would be minimal or absent. However, other heavy metals could aid in the transition of *P. aeruginosa* into the VBNC state in drinking water, which in sum could be present in concentrations affecting *P. aeruginosa* culturability. A potential candidate known for its toxicity is Zn, which could influence *P. aeruginosa* culturability in combination with Cu (Teitzel and Parsek, 2003). Results of this study, similarly to previous studies comparing culture dependent to culture independent methods, indicate that under unfavorable conditions, culture dependent methods may underestimate the presence of hygienically relevant microorganisms and, thus, potential risk to human health.

The effect of a simulated contamination of drinking-water biofilms with *P. aeruginosa* on the biochemical composition of the biofilms was determined by analyzing EPS composition 24 h and 7 d after inoculation of the biofilms with *P. aeruginosa* AdS. The quantitative biochemical composition of total biofilms and the EPS showed only slight variations. Analysis of the extracellular proteome revealed a small number of differentially produced EPS proteins. Concluding, exposure of drinking-water biofilms to *P. aeruginosa* in order to simulate a contamination of drinking water with a hygienically relevant microorganism, showed no significant alterations to the community or biochemical composition of the

drinking-water biofilm. *P. aeruginosa* detected by FISH made up only 0.04 % to 0.05 % of the total cell counts and were probably too few to produce significant alteration to the established drinking-water biofilms. Slight variations could have been attributed by biofilm to biofilm alterations, rather than the influence of *P. aeruginosa*.

The consequence of an incorporation of the facultative pathogen into drinking-water biofilms, regarding hygienical relevance and potential threat to human health, remains subject to speculation. The potential of *P. aeruginosa* to be incorporated into drinking-water biofilms and its persistence in the biofilm environment has been shown, however, the pathogen's physiological state remains unknown. In the present study *P. aeruginosa* is assumed to be in a VBNC state when incorporated into drinking-water biofilms, due to toxic concentrations of heavy metals, in particular Cu, in the drinking water (Moritz *et al.*, Dwidjosiswojo *et al.*, 2011). The environmental conditions within a biofilm, however, are drastically altered compared to the water phase, and can be advantageous or disadvantageous to the pathogen. Biofilms in general are known to provide a safe haven for microorganisms, due to their protective functions (Hall-Stoodley *et al.*, 2004). However, in this study drinking-water biofilms were shown to sequester and concentrate heavy metals within the biofilm matrix by several orders of magnitude compared to the water phase. Despite their potential complexation by EPS components or deposition as insoluble precipitates in the biofilm matrix, which could alter their toxicity (Gadd, 2010), their effects on *P. aeruginosa* within the biofilm matrix are unknown. Additionally, *P. aeruginosa* could encounter antagonistic microorganisms, which have been described in marine environments (Nithya *et al.*, 2010; Rendueles and Ghigo, 2012), thus, increasing hostility of the biofilm environment for *P. aeruginosa* and decreasing its chances of survival. The question what happens, once incorporated *P. aeruginosa* are released from the biofilm into the bulk solution, remains to be investigated.

5.4 Identification and function of extracellular proteins in drinking-water biofilms

Proteins in the EPS matrix are considered to have their major functions as enzymes, allowing for metabolic turnover of diverse substrates accumulated in the EPS matrix, or as structural components, providing mechanical stability to the biofilm (Wingender and Jaeger, 2002; Flemming and Wingender, 2010). Despite their profound role in the EPS matrix of biofilms, identification of extracellular proteins has been performed only in a small number of studies found in literature, dealing with pure culture biofilms. Identification of EPS proteins from drinking-water biofilms has never been attempted before.

For identification and in order to elucidate the function of EPS proteins present in drinking-water biofilms, EPS were isolated from the biofilms by use of CER and subjected to separation by 2DE and subsequent analysis by MALDI-TOF-MS. 2DE separates proteins according to their isoelectric point (pI) in the first dimension and by their molecular weight in the second dimension, and in an ideal case provides spot patterns with each spot corresponding to a single protein. The EPS of drinking-water biofilms required additional clean-up and optimization of the 2DE protocol in order to obtain distinct protein spots, which was achieved in this study.

In the present study, identification of protein spots in most of the cases revealed significant similarity of MS-spectra to more than one protein. The 2DE gels produced for subsequent identification of proteins showed horizontal and vertical streaking, due to the increased amount of proteins (400 µg) applied for 2D separation and potential overloading of the IPG gels (Garfin and Heerdt, 2000). A high protein concentration was required for identification to ensure sufficient protein amount per protein spot. Consequentially, separation of proteins was not ideal and could have resulted in presence of multiple proteins per spot. Alternatively, MS-spectra of analyzed proteins may have close homology to a variety of proteins present in the database, resulting in multiple identifications for a single protein. Information on molecular weight and estimated pI were examined as potential means to validate protein identity. However, molecular weight and estimated pI of the identified proteins only in a few cases corresponded to the location of the proteins on the gels. In many cases molecular weight of identified protein was significantly higher compared to the

location on the 2DE gel. This could indicate that only subunits of these proteins or enzymatically degraded proteins were detected by 2DE. In some cases molecular weight of identified protein was lower compared to its location on the 2DE gel. This may be attributed to interactions of EPS components with proteins or their interference with 2DE. Polysaccharides and DNA are known to disturb isoelectric focusing by clogging the pores of the gels (Berkelman *et al.*, 2000; Berkelman, 2008). Furthermore, EPS components can bind to or entangle proteins (Wloka *et al.*, 2004; Berkelman *et al.*, 2000; Berkelman 2008; Flemming and Wingender, 2010). The EPS solutions applied for 2DE in this study contained considerable amounts of polysaccharides and potentially lipids. Associations of proteins with other EPS components can influence the proteins' mobility during 2DE and could have substantially altered molecular weight and pI of EPS proteins visualized by 2DE in this study. Consequentially, information on molecular weight and estimated pI could not be used as means to help in the validation of the identity of proteins.

Allocation of proteins was predicted from their amino acid sequence. The majority of identified proteins were predicted as intracellular or cell membrane associated. The presence of intracellular proteins in the EPS may indicate lysis of cells in the course of biofilm formation. Cell death and consequential lysis of cells allows for release of intracellular material into the environment as response to environmental stress and is considered a necessary element during the formation of biofilms (Rice *et al.*, 2007; O'Connell, 2007). Resulting discharge of intracellular proteins could have resulted in the retention and implementation of these proteins within the EPS matrix of drinking-water biofilms in the present study. Intracellular proteins have also been detected in the EPS of *Bacillus subtilis* biofilms (Hirose *et al.*, 2000), further indicating that intracellular proteins are generally present in the EPS matrix. Cell lysis induced by the EPS isolation procedure by CER was not detected by means of culturability or determination of activity of the strictly intracellular enzyme G6PDH in the EPS. Contributions of intracellular proteins released due to CER treatment are, therefore, considered minimal. The presence of membrane associated proteins may also be attributed to lysis of cells in the course of biofilm development. Alternatively, production of membrane vesicles, which has been shown for a variety of Gram-negative bacteria (reviewed by Kuehn and Kesty, 2005) could contribute to the presence of outer membrane proteins in the EPS matrix. In the case of membrane-bound

proteins, in particular those located in the outer membrane, the EPS isolation treatment by CER needs to be considered. Membrane-bound proteins are exposed to the shear imposed by the CER treatment. Presence of membrane-associated proteins in the EPS might indicate their shedding from the outer membrane during EPS isolation by CER, with no noticeable effect on the cells' viability. A total of 11 proteins present in the NCBI database, which were predicted to be localized extracellularly, exhibited significant homology of MS-spectra to proteins present in the EPS of drinking-water biofilms.

Identification of EPS proteins from drinking-water biofilms indicated presence of proteins with diverse functions. The majority of proteins belonged to the functional classes involved in metabolism, regulation or transport. These functions have also been described for EPS proteins of pure culture biofilms of *B. subtilis* (Hirose *et al.*, 2000; Antelmann *et al.*, 2001), *E. coli* (Nandakumar *et al.*, 2005) and *P. aeruginosa* (Nouvens *et al.*, 2003; Kim *et al.*, 2005; Waite *et al.*, 2012).

The identification results suggest presence of a variety of hydrolytic enzymes involved in metabolic functions, which are predicted to be cell-associated (e.g. spots no. 12, 18, 23, 26, 34, 35), or extracellularly localized (e.g. spot no. 6). The identified enzymes belong to the enzyme groups proteases, peptidases, phosphatases, nucleases, kinases, or oxidoreductases. Potential metabolic capabilities of drinking-water biofilms have been discussed by Schmeisser *et al.* (2003), based on metagenome information obtained from drinking-water biofilms on rubber coated valves in a drinking water distribution system, who matched obtained gene sequences to known protein-coding sequences. Enzymatic activity measurements (Section 4.4.6) and EPS protein identification results of the present study further indicate the metabolic potential of drinking-water biofilms and, in particular, the EPS' role as an external digestive system.

Similarity of MS-spectra to response regulatory proteins (spots no. 3, 9, 24, 32) or sensory proteins (spots no. 3, 27) has been detected for a number of EPS proteins from drinking-water biofilms. These proteins respond to environmental stimuli and effect stress response under adverse conditions. In the presented study environmental stress on microorganisms has been described in the form of Cu stress. Spots No. 3 and 32 have been found in clusters of protein spots present only in the EPS of drinking-water biofilms cultivated in drinking-

waters abstracted from plumbing systems made of Cu, and absent in those cultivated at Cu-unaffected distribution systems. The gene sequence encoding for a two-component response regulator protein, which had a significant homology of MS-spectrum to protein spot No. 32 analyzed in this study, was found in an uncultured bacterium from a drinking-water biofilm (Stoeckigt *et al.*, unpublished; accession no. Q6JWS4). Presence of these proteins in biofilms exposed to elevated concentrations of Cu indicates their significance as regulator of gene expression towards enhanced production of Cu stress specific cell components and EPS.

Transporter systems are essential for cell viability, importing nutrients from the environment and exporting substances toxic to the cell (Davidson *et al.*, 2008). Transporter proteins are, furthermore, necessary for the establishment of a mature biofilm, due to their role in translocation of polymers from the cytoplasm into the extracellular environment (Hinsa *et al.*, 2003). Secretory systems involve complex arrangements of numerous secretory proteins bound to the inner or outer cell membrane or spanning across the periplasm, and which form specific pathways for the transport of polymers across the membranes (Hueck, 1998; Russel, 1998; Abdallah *et al.*, 2007; Fronzes *et al.*, 2009). A number of proteins analyzed in the present study showed significant homology in MS-spectra to transporter proteins (spots no. 5, 6, 7, 9, 13, 16, 18, 27, 31). Regarding spot no. 9, this protein was only found in drinking-water biofilms exposed to elevated concentrations of Cu, and therefore, could be involved in the Cu stress response mechanism. The majority of transporter proteins detected in this study were predicted to be located in the outer membrane.

Proteins can also exhibit structural roles within the biofilm matrix (Higgins and Novak, 1997; Dignac *et al.*, 1998; Tielker *et al.*, 2005; Larsen *et al.*, 2007). Identification results indicated low abundance of structural proteins in the EPS of drinking-water biofilms, showing only one potential candidate (spot no. 6) with significant homology in MS-spectrum to laminin, beta 3. A further type of proteins detected in the EPS of *E. coli*, *B. subtilis* and *P. aeruginosa* included (Antelmann *et al.*, 2001; Nouvens *et al.*, 2003; Kim *et al.*, 2005; Nandakumar *et al.*, 2006; Waite *et al.*, 2012) are flagellar proteins. These proteins were not found in the EPS of drinking-water biofilms in the present study. For a number of proteins analyzed in this study, including 4 proteins which were predicted to be localized extracellularly, the function is unknown.

5.5 Outlook

The presented study provided insights into the composition, function and dynamics of drinking-water biofilms and their EPS. The major challenge of this study was the low concentration of the EPS, and thus, the establishment and adaptation of methods for the cultivation of drinking-water biofilms, for EPS isolation, as well as methods for quantitative and qualitative analyses of the biofilms and their EPS.

The experimental setup and EPDM material applied in this study proved to be suitable for the cultivation of drinking-water biofilms, supporting extensive growth of biofilms and resulting in macroscopically visible, multi-layered biofilms. Biofilm growth was supported by nutrients leached from the EPDM material. Such extensive growth was necessary in this study, to obtain sufficient biomass for EPS isolation and analysis. In the drinking water praxis such an extensive biofilm formation would represent a worst-case scenario. However, materials which support microbial growth are still used, mainly in plumbing systems. Therefore, the experimental system has practical relevance.

The EPS isolation method by CER, which was miniaturized and optimized in the present study, allowed for recovery of increased amounts of EPS from drinking-water biofilms and for a broad range of biochemical analyses. Alternative procedures for EPS isolation, as reported in literature, in particular chemical methods, showed limitations in terms of interference with biochemical assays, potential cell lysis or chemical reactions with EPS constituents.

Drinking-water biofilms were shown to be another type of biofilm, in which proteins represent the main EPS component, irrespective of biofilm age and origin, followed by polysaccharides and DNA. Prevalence of proteins has also been observed, e.g. in wastewater biofilms. Establishment and optimization of analytical methods in the present study enabled the determination of the role of extracellular proteins within drinking-water biofilms. EPS proteins exhibited enzymatic functions and were shown to utilize a variety of diverse substrates, indicating their role as an external digestive system. The optimized 2DE protocol allowed for separation and subsequent identification of EPS proteins, which showed metabolic, transport or regulatory functions. However, identification of proteins separated

by 2DE and analyzed by MALDI-TOF-MS in most cases resulted in significant homologies of MS-spectra to more than one protein recorded in the protein database per spot. Recently developed approaches, such as the combination of metagenomics and proteomics, represent potential tools to obtain more exact identifications of EPS proteins from drinking-water biofilms as well as other environmental multi-species biofilms. This approach could help to exclude improbable identities, and should be applied in future studies. Application of 2D-DIGE could, furthermore, allow for more precise detection of up- or down-regulation of intra- or extracellular protein expression in drinking-water biofilms in response to environmental changes or stress.

Polysaccharides and DNA were present in considerable proportions in the EPS of drinking-water biofilms. Polysaccharides are structural elements of any biofilm and for a long time considered the major constituent of biofilms. In this study polysaccharides were only analyzed quantitatively. Qualitative analysis of the composition and structure of polysaccharides will be necessary to determine the influence of polysaccharides on biofilm structure and mechanical stability. Presence of eDNA was verified in all drinking-water biofilms tested in this study, indicating its ubiquity in this type of biofilms. This raises the question of its role within the EPS matrix. Whitchurch *et al.* (2002) demonstrated, that eDNA is a structural component within the EPS of *P. aeruginosa* biofilms, and not merely debris from lysed cells. Flemming *et al.* (2007) speculated about further potential functions of eDNA, which have yet to be proven. The role of eDNA within drinking-water biofilms remains subject to future investigations.

Finally, this study demonstrated the variability of drinking-water biofilms in terms of microbial populations and EPS composition in the progress of biofilm formation, as well as in response to differences in conditions among drinking water systems. Variations were in particular evident comparing biofilms from distribution systems to those cultivated in copper plumbing systems, with regard to population diversity and EPS composition. Copper was shown to influence the culturability of some microorganisms and may be responsible for population shifts within drinking-water biofilms. Furthermore, copper was shown to decrease culturability of *P. aeruginosa,* which was used as model organism of hygienical relevance, causing its transition into the VBNC state and potentially obstructing its detection

during drinking water analysis. This signifies the need for further investigations of the effect of copper on drinking-water biofilms and on hygienically relevant organisms.

6. REFERENCES

Abdallah, A. M., Gey van Pittius, N. C., DiGiuseppe Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M. J. E., Appelmelk, B. J., Bitter, W. (2007). Type VII secretion - mycobacteria show the way. Nature Reviews Microbiology 5, 883 – 891.

Aguilera, A., Souza-Egipsy, V., San Martín-Úriz, P., Amils, R. (2008). Extraction of extracellular polymeric substances from extreme acidic microbial biofilms. Applied Microbiology and Biotechnology 78, 1079 – 1088.

Ahn, S. J., Costa, J., Emanuel, J. R. (1996). PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acid Research 24, 2623 – 2625.

Akpe San Roman, S., Facey, P. D., Fernandez-Martinez, L., Rodriguez, C., Vallin, C., Del Sol, R., Dyson, P. (2010). A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in *Streptomyces coelicolor*. Microbiology 156, 1719 – 1729.

Alexander, E., Pham, D., Steck, T. R. (1999). The viable-but-nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. Applied and Environmental Microbiology 65, 3754 – 3756.

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., Tolker-Nielsen, T. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Molecular Microbiology 59, 1114 – 1128.

Allison, D. G., Sutherland, I. W. (1984). A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. Journal of Microbiological Methods 2, 93 – 99.

Allison, D. P., Kerper, P. S., Doktycz, M. J., Thundat, T., Modrich, P., Larimer, F. W., Johnson, D. K., Hoyt, P. R., Mucenski, M. L., Warmack, R. J. (1997). Mapping individual cosmid DNAs by direct AFM imaging. Genomics 41, 379 – 384.

Allison, D. G., Ruiz, B., SanJose, C., Jaspe, A., Gilbert, P. (1998). Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Microbiology Letters 167, 179 – 184.

Amann, R. I., Ludwig, W., Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiology and Molecular Biology Reviews 59, 143 – 169.

Angles, M. L., Marshall, K. C., Goodman, A. E. (1993). Plasmid transfer between marine bacteria in the aqueous phase and biofilms in reactor microcosms. Applied and Environmental Microbiology 59, 843 – 850.

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijl, J. M., Hecker, M. (2001). A proteomic view on genome-based signal peptide predictions. Genome Research 11, 1484 – 1502.

Appenzeller, B. M. R., Batté, M., Mathieu, L., Block, J.-C., Lahoussine, V., Cavard, J., Gate, D. (2001). Effect of adding phosphate in drinking water on bacterial growth in slightly and highly corroded pipes. Water Research 35, 1100 – 1105.

Artz, R. R. E., Killham, K. (2002). Survival of *Escherichia coli* O157:H7 in private drinking water wells: influences of protozoan grazing and elevated copper concentrations. FEMS microbiology letters 216, 117 – 122.

Azeredo, L., Lazarova, V., Oliveira, R. (1999). Methods to extract the exopolymeric matrix from biofilms, a comparative study. Water Science and Technology 39(7), 243 – 250.

Azeredo, J., Henriques, M., Sillankorva, S., Oliveira, R. (2003). Extraction of exopolymers from biofilms: the protective effect of glutaraldehyde. Water Science and Technology 47(5), 175 – 179.

Bagh, L. K., Albrechtsen, H.-J., Arvin, E., Ovesen, K. (2004). Distribution of bacteria in a domestic hot water system in a Danish apartment building. Water Research 38, 225 - 235.

Bai, X., Wu, F., Zhou, B., Zhi, X. (2010). Biofilm bacterial communities and abundance in a full-scale drinking water distribution system in Shanghai. Journal of Water and Health 8, 593-600.

Barbesti, S., Citterio, S., Labra, M., Baroni, M. D., Neri, M. G., Sgorbati, S. (2000). Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. Cytometry 40, 214 – 218.

Batté, M., Appenzeller, B. M. R., Grandjean, D., Fass, S., Gauthier, V., Jorand, F., Mathieu, L., Boualam, M., Saby, S., Block, J. C. (2003). Biofilms in drinking water distribution systems. Reviews in Environmental Science and Biotechnology 2, 147 – 168.

Battin, T. J. (1997). Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms. Science of the Total Environment 198, 51 – 60.

Baty III, A. M., Eastburn, C. C., Diwu, Z., Techkarnjanaruk, S., Goodman, A. E., Geesey, G. G. (2000). Differentiation of chitinase-active and non-chitinase-active subpopulations of a marine bacterium during chitin degradation. Applied and Environmental Microbiology 66, 3566 – 3573.

Baty III, A. M., Diwu, Z., Dunham, G., Eastburn, C. C., Geesey, G. G., Goodman, A. E., Suci, P. A., Techkarnjanaruk, S., (2001). Characterization of extracellular chitinolytic activity in biofilms. Methods in Enzymology 336, 279 – 301.

Beccari, M., Mappelli, P., Tandoi, V. (1980). Relationship between bulking and physicochemical-biological properties of activated sludge. Biotechnology and Bioengineering 12, 969 – 979.

Beech, I. B. (1996). The potential use of atomic force microscopy for studying corrosion of metals in the presence of bacterial biofilms — an overview. International Biodeterioration and Biodegradation 37, 141 – 149.

Beech, I. B., Smith, J. R., Steele, A. A., Penegar, I., Campbell, S. A. (2002). The use of atomic force microscopy for studying interactions of bacterial biofilms with surfaces. Colloids and Surfaces B, Biointerfaces 23, 231 – 247.

Ben-Amor, K., Heilig, H., Smidt, H., Vaughan, E. E., Abee, T., de Vos, W. M. (2005). Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. Applied and Environmental Microbiology 71, 4679 – 4689.

Berkelman, T., Brubacher, M. G., Chang, H., Cross, T., Strong, W. (2000). Tips to prevent streaking on 2-D gels. Bio-Rad bulletin 3110.

Berkelman, T. (2008). Removal of interfering substances in samples prepared for two-dimensional (2-D) electrophoresis. Methods in Molecular Biology 424, 51 – 62.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., Egli, T. (2007). Assessment and interpretation of bacterial viability by using the Live/Dead® *Bac*Light kit in combination with flow cytometry. Applied and Environmental Microbiology 73, 3283 – 3290.

Berry, D., Xi, C., Raskin, L. (2006). Microbial ecology of drinking water distribution systems. Current Opinion in Biotechnology 17, 297 – 302.

Beveridge, T. J., Makin, S. A., Kadurugamuwa, J. L., Li, Z. (1997). Interactions between biofilms and the environment. FEMS Microbiology Reviews 20, 291 – 303.

Birmingham, J. J., Hughes, N. P., Treloar, R. (1995). Diffusion and binding measurement within oral biofilms using fluorescence photobleaching recovery methods. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 350, 325 – 343.

Bliss, J. M., Silver, R. P. (1996). Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. Molecular Microbiology 21, 221 – 231.

Block, J. C., Haudidier, K., Paquin, J. L., Miazga, J. and Levi, Y. (1993). Biofilm accumulation in drinking water distribution systems. Biofouling 6, 333 – 343.

Blumenkrantz, N., Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. Analytical Biochemistry 54, 484 – 489.

Böckelmann, U., Manz, W., Neu, T. R., Szewzyk, U. (2000). Characterization of the microbial community of lotic organic aggregates ("river snow") in the Elbe River of Germany by cultivation and molecular methods. FEMS Microbiology Ecology 33, 157 – 170.

Böckelmann, U., Janke, A., Kuhn, R., Neu, T. R., Wecke, J, Lawrence, J. R., Szewzyk, U. (2006). Becterial Extracellular DNA forming a defined network-like structure. FEMS Microbiology Letters 262, 31 – 38.

Böckelmann, U., Lünsdorf, H., Szewzyk, U. (2007). Ultrastructural and electron energy-loss spectroscopic analysis of an extracellular filamentous matrix of an environmental bacterial isolate. Environmental Microbiology 9, 2137 – 2144.

Boe-Hansen, R., Albrechtsen, H.-J., Arvin, E., Jørgensen, C. (2002). Dynamics of biofilm formation in a model drinking water distribution system. Journal of Water Supply: Research and Technology - AQUA 51, 399 – 406.

Bonner, O. D., Smith, L. L. (1957). A selectivity scale for some divalent cations on Dowex 50. Journal of Physical Chemistry 61, 326 – 329.

Boschker, H. T., Cappenberg, T. (1998). Patterns of extracellular enzyme activities in littoral sediments of Lake Gooimeer, The Netherlands. FEMS Microbiology Ecology 25, 79 – 86.

Boyd, A., Chakrabarty, A. M. (1994). Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 60, 2355 – 2359.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248 – 254.

Brecker, L., Ribbons, D. W. (2000). Biotransformations monitored *in situ* by proton nuclear magnetic resonance spectroscopy. Trends in Biotechnology 18, 197 – 202.

Breeveld, M. W., Zevenhuizen, T. M., Zehnder, A. J. B. (1990). Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* Su47. Journal of General Microbiology 136, 2511 – 2519.

Bressler, D. (2008). Einnistung von *Pseudomonas aeruginosa* in Trinkwasserbiofilme: Eine Frage der EPS? Dissertation, University of Duisburg-Essen, Germany.

Bressler, D., Balzer, M., Dannehl, A., Flemming, H.-C., Wingender, J., (2009). Persistence of *Pseudomonas aeruginosa* in drinking-water biofilms on elastomeric material. Water Science Technology: Water Supply 9, 81 – 87.

Broekman, S. (2009). Etablierung der Methodik zur Darstellung des extrazellulären Proteoms in Biofilmen eines mucoiden Stammes von *Pseudomonas aeruginosa*. Dissertation, University of Duisburg-Essen, Germany.

Brown, M. J., Lester, J. N. (1980). Comparison of bacterial extracellular polymer extraction methods. Applied and Environmental Microbiology 40, 179 – 185.

Brunk, C., Jones, K. C., James, T. W. (1979). Assay for nanogram quantities of DNA in cellular homogenates. Analytical Biochemistry 92, 497 – 500.

Buckmire, F. L. A. (1984). Influence of nutrient media on the characteristics of the exopolysaccharide produced by three mucoid *Pseudomonas aeruginosa* strains. Microbios 41, 49 – 63.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. The Biochemical Journal 62, 315 – 323.

Busch, P. L., Stumm, W. (1968). Chemical interactions in the aggregation of bacteria bioflocculation in waste treatment. Environmental Science and Technology 2, 49 – 53.

Caldwell, D. E., Korber, D. R., Lawrence, J. R. (1992). Confocal laser scanning microscopy and digital image analysis in microbial ecology. Advances in Microbial Ecology 12, 1 – 67.

Cao, B., Shi, L., Brown, R. N., Xiong, Y., Fredrickson, J. K., Romine, M. F., Marshall, M. J., Lipton, M. S., Beyenal, H. (2011). Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics. Environmental Microbiology 13, 1018 – 1031.

Catlin, B. W. (1956). Extracellular deoxyribonucleic acid of bacteria and a deoxyribonuclease inhibitor. Science 124, 441 – 442.

Cegelski, L., Pinkner, J. S., Hammer, N. D., Cusumano, C. K., Hung, C. S., Chorell, E., Åberg, V., Walker, J. N., Seek, P. C., Almqvist, F., Chapman, M. R., Hultgren, S. J. (2009). Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nature Chemical Biology 5, 913 – 919.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. Journal of Bacteriology 183, 5385 – 5394.

Chang, Y. C., Puil, M. L., Biggerstaff, J., Randall, A. A., Schulte, A., Taylor, J. S. (2003). Direct estimation of biofilm density on different pipe material coupons using a specific DNA-probe. Molecular and Cellular Probes 17, 237 – 243.

Chapman, M. R., Robinson, L. S., Pinkner, J. S., Roth, R., Heuser, J., Hammar, M., Normark, S., Hultgren, S. J. (2002). Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science 295, 851 – 855.

Characklis, W. G., Wilderer, P. A. (1989). Structure and function of biofilms. John Wiley & Sons, Inc., Chichester.

Chen, G., Palmer, R. J., White, D. C. (1997). Instrumental analysis of microbiologically influenced corrosion. Biodegradation 8, 189 – 200.

Chen, C. H., Clegg, D. O., Hansma, H. G. (1998). Structures and dynamic motion of laminin-1 as observed by atomic force microscopy. Biochemistry 37, 8262 – 8267.

Chen, L., Hu, B., Qian, G., Wang, C., Yang, W., Han, Z., Liu, F. (2009). Identification and molecular characterization of twin-arginine translocation system (Tat) in *Xanthomonas oryzae* pv. *oryzae* strain PXO99. Archives of Microbiology 191, 163 – 170.

Choo-Smith, L.-P., Maquelin, K., van Vreeswijk, T., Bruining, H. A., Puppels, G. J., Ngo Thi, N. A., Kirschner, C., Naumann, D., Ami, D., Villa, A. M., Orsini, F., Doglia, S. M., Lamfarraj, H., Sockalingum, G. D., Manfait, M., Allouch, P., Endtz, H. P. (2001). Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy. Applied and Environmental Microbiology 67, 1461 – 1469.

Christensen, B. E., Kjosbakken, J., Smidsrød, O. (1985). Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. strain NCMB 2021. Applied and Environmental Microbiology 50, 837 – 845.

Chung, Y.-C., Neethling, J. B. (1989). Microbial activity measurements for anaerobic sludge digestion. Journal of the Water Pollution Control Federation 61, 343 – 349.

Comte, S., Guibaud, G., Baudu, M. (2006a). Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties. Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme and Microbial Technology 38, 237 – 245.

Comte, S., Guibaud, G., Baudu, M. (2006b). Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part II. Consequences of EPS extraction methods on Pb^{2+} and Cd^{2+} complexation. Enzyme and Microbial Technology 38, 246 – 252.

Comte, S., Guibaud, G., Baudu, M. (2008). Biosorption properties of extracellular polymeric substances (EPS) towards Cd, Cu and Pb for different pH values. Journal of Hazardous Materials 151, 185 – 93.

Confer, D. R., Logan, B. E. (1998). Location of protein and polysaccharide hydrolytic activity in suspended and biofilm wastewater cultures. Water Research 32, 31 – 38.

Conrad, A., Suutari, M. K., Keinänen, M. M., Cadoret, A., Faure, P., Mansuy-Huault, L., Block, J. C. (2003). Fatty acid lipid fractions in extracellular polymeric substances of activated sludge. Lipids 38, 1093 – 1105.

Conti, E., Flaibani, A., O'Regan, M., Sutherland, I. W. (1994). Alginate from *Pseudomonas fluorescens* and *P. putida*: production and properties. Microbiology 140, 1125 – 1132.

Cooksey, D. A. (1994). Molecular mechanisms of copper resistance and accumulation in bacteria. FEMS Microbiology Reviews 14, 381 – 386.

Costerton, J. W., Geesey, G. G., Cheng, G. K. (1978). How bacteria stick. Scientific American 238, 86 – 95.

Costerton, J. W., Irvin, R. T., Cheng, K.-J. (1981). The bacterial glycocalyx in nature and disease. Annual Reviews in Microbiology 35, 299 – 324.

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., Marrie, T. J. (1987). Bacterial biofilms in nature and disease. Annual Review of Microbiology 41, 435 – 464.

Costerton, J. W. (1995). Overview of microbial biofilms. Journal of Industrial Microbiology 15, 137 – 140.

D'Abzac, P., Bordas, F., Joussein, E., van Hullebusch, E., Lens, P. N. L., Guibaud, G. (2010). Characterization of the mineral fraction associated to extracellular polymeric substances (EPS) in anaerobic granular sludges. Environmental Science and Technology 44, 412 – 418.

Danilatos, G. D. (1981). Design and construction of an atmospheric or environmental SEM (Part 1). Scanning, 4, 9 – 20.

Danilatos, G. D. (1983). A gaseous detector for an environmental SEM. Micron and Microscopica Acta 14, 307 – 318.

Das, T., Sharma, P. K., Busscher, H. J., van der Mei, H. C., Krom, B. P. (2010). Role of extracellular DNA in initial bacterial adhesion and surface aggregation. Applied and Environmental Microbiology 76, 3405 – 3408.

Davey, M. E., O'Toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews 64, 847 – 867.

Davidson, A. L., Dassa, E., Orelle, C., Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiology and Molecular Biology Reviews 72, 317 – 364.

Dazzo, F. B., Wright, S. F. (1996). Production of anti-microbial antibodies and their use in immunofluorescence microscopy. In: Molecular microbial ecology manual, 4.1.2, pp. 1 – 27. Akkermans, A. D. L., van Elsas, J. D., de Bruijn, F. J. (Eds.), Kluwer, Dordrecht.

de Beer, D., Stoodley, P., Roe F., Lewandowski, Z. (1994). Effects of biofilm structures on oxygen distribution and mass transport. Biotechnology and Bioengineering 43, 1131 – 1138.

De Rosa, S., Sconza, F., Volterra, L. (1998). Biofilm amount estimation by fluorescein diacetate. Water Research 32, 2621 – 2626.

Decho, A. W., Lopez, G. R. (1993). Exopolymer microenvironments of microbial flora: multiple and interactive effects on trophic relationships. Limnology and Oceanography 38, 1633 – 1645.

Decho, A. W. (2000). Microbial biofilms in intertidal systems: an overview. Continental Shelf Research 20, 1257 – 1273.

Declerck, P., Behets, J., Margineanu, A., van Hoef, V., De Keersmaecker, B., Ollevier, F. (2009). Replication of *Legionella pneumophila* in biofilms of water distribution pipes. Microbiology Research 164, 593 – 603.

Deines, P., Sekar, R., Husband, P. S., Boxall, J. B., Osborn, A. M., Biggs, C. A. (2010). A new coupon design for simultaneous analysis of *in situ* microbial biofilm formation and community structure in drinking water distribution systems. Applied Microbiology and Biotechnology 87, 749 – 756.

Del Campo, R., Russi, P., Mara, P., Mara, H., Peyrou, M., Ponce de León, I., Gaggero, C. (2009). *Xanthomonas axonopodis* pv. *citri* enters the VBNC state after copper treatment and retains its virulence. FEMS Microbiology Letters 298, 143 – 148.

Denkhaus, E., Meisen, S., Telgheder, U., Wingender, J. (2007). Chemical and physical methods for characterisation of biofilms. Microchim Acta 158, 1 – 27.

Dey, E. S., Szewczyk, E., Wawrzynczyk, J., Norrlöw, O. (2006). A novel approach for characterization of exopolymeric material in sewage sludge. Journal of Residuals Science and Technology 3, 97 – 103.

Di Poto, A., Sbarra, M. S., Provenza, G., Visai, L., Speziale, P. (2009). The effect of photodynamic treatment combined with antibiotic action or host defence mechanisms on Staphylococcus aureus biofilms. Biomaterials 30, 3158 – 3166. Elsevier Ltd.

Dignac, M. F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D., Scribe, P. (1998). Chemical description of Extracellular polymers: implication on activated sludge floc structure. Water Science and Technology 38(8-9), 45 – 53.

Dillard, J. P., Seifert, H. S. (2001). A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. Molecular Microbiology 41, 263 – 277.

Domek, M. J., LeChevallier, M. W., Cameron, S. C., McFeters, G. A. (1984). Evidence for the role of copper in the injury process of coliform bacteria in drinking water. Applied and Environmental Microbiology 48, 289 – 293.

Dominiak, D. M., Nielsen, J. L., Nielsen, P. H. (2011). Extracellular DNA is abundant and important for microcolony strength in mixed microbial biofilms. Environmental Microbiology 13, 710 – 721.

Donlan, R. M., Costerton, J. W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews 15, 167 – 193.

Drummelsmith, J., Whitfield, C. (2000). Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* requires a multimeric complex in the outer membrane. The EMBO Journal 19, 57 – 66.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350 – 356.

DVGW (2007). Technische Regel W 270. Vermehrung von Mikroorganismen auf Werkstoffen für den Trinkwasserbereich – Prüfung und Bewertung. DVGW, Bonn.

Dwidjosiswojo, Z., Richard, J., Moritz, M. M., Dopp, E., Flemming, H.-C., Wingender, J. (2011). Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa* under conditions relevant to drinking water environments. International Journal of Hygiene and Environmental Health 214, 485 – 492.

Eboigbodin, K. E., Biggs, C. A. (2008). Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies. Biomacromolecules 9, 686 – 695.

Eboigbodin, K. E., Seth, A., Biggs, C. A. (2008). A review of biofilms in domestic plumbing. Journal AWWA 100, 131 – 138.

Eichler, S., Christen, R., Holtje, C., Westphal, P., Botel, J., Brettar, I., Mehling, A., Höfle, M. G. (2006). Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting. Applied and Environvironmental Microbiology 72, 1858 – 1872.

Elfström Broo, A., Berghult, B., Hedberg, T. (2001). Pipe material selection in drinking water systems - a conference summary. Water Science Technology: Water Supply 1, 117 – 125.

Elguindi, J., Moffitt, S., Hasman, H., Andrade, C., Raghavan, S., Rensing, C. (2011). Metallic copper corrosion rates, moisture content, and growth medium influence survival of copper ion-resistant bacteria. Applied Microbiology and Biotechnology 89, 1963 – 1970.

Ellen, A. F., Albers, S.-V., Huibers, W., Pitcher, A., Hobel, C. F. V., Schwarz, H., Folea, M., Schouten, S., Boekema, E. J., Poolman, B., Driessen, A. J. M. (2009). Proteomic analysis of secreted membrane vesicles of archaeal Sulfolobus species reveals the presence of endosome sorting complex components. Extremophiles 13, 67 – 79.

Emde, K. M. E., Smith, D. W., Facey, R. (1992). Initial investigation of microbially influenced corrosion (MIC) in a low temperature water distribution system. Water Research 26, 169 – 175.

Emtiazi, F., Schwartz, T., Marten, S. M., Krolla-Sidenstein, P., Obst, U. (2004). Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. Water Research 38, 1197 – 1206.

Engelberg-Kulka, H., Hazan, R. (2003). Cannibals defy starvation and avoid sporulation. Science 301, 467 – 468.

Engelberg-Kulka, H., Amitai, S., Kolodkin-Gal, I., Hazan, R. (2006). Bacterial programmed cell death and multicellular behavior in bacteria. PLoS Genetics 2, e135.

Erni, R., Rossell, M. D., Kisielowski, C., Dahmen, U. (2009). Atomic-resolution imaging with a sub-50-pm electron probe. Physical Review Letters 102, 096101.

Espeland, E. M., Wetzel, R. G. (2001). Effects of photosynthesis on bacterial phosphatase production in biofilms. Microbial Ecology 42, 328 – 337.

Evans, E., Brown, M. R. W., Gilbert, P. (1994). Iron chelator, exopolysaccharide and protease production in *Staphylococcus epidermidis*: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. Microbiology 140, 153 – 157.

Fang, H. P., Jia, X. S. (1996). Extraction of extracellular polymers from anaerobic sludges. Biotechnology Techniques 10, 803 – 808.

Farag, A. M., Nimick, D. A., Kimball, B. A., Church, S. E., Harper, D. D., Brumbaugh, W. G. (2007). Concentrations of metals in water, sediment, biofilm, benthic macroinvertebrates, and fish in the Boulder River watershed, Montana, and the role of colloids in metal uptake. Archives of Environmental Contamination and Toxicology 52, 397 – 409.

Farrah, S. R., Unz, R. F. (1976). Isolation of exocellular polymer from *Zoogloea* strains MP6 and 106 and from activated sludge. Applied and Environmental Microbiology 32, 33 – 37.

Filisetti-Cozzi, T.M., Carpita, N.C. (1991). Measurement of uronic acids without interference of neutral sugars. Analytical Biochemistry 197, 157 – 162.

Fiume, L., Bucci Sabattini, M. A., Poda, G. (2005). Detection of *Legionella pneumophila* in water samples by species-specific real-time and nested PCR assays. Letters in Applied Microbiology 41, 470 – 475.

Flemming, H.-C. (1998). Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. Polymer Degradation and Stability 59, 309 – 315.

Flemming, H.-C., Wingender, J. (2001). Biofilme - die bevorzugte Lebensform der Bakterien: Flocken, Filme und Schlämme. Biologie in unserer Zeit 31, 169 – 180.

Flemming, H.-C. (2002). Biofouling in water systems - cases, causes, countermeasures. Applied Microbiology and Biotechnology 59, 629 – 640.

Flemming, H.-C., Percival, S., Walker, J. T. (2002). Contamination potential of biofilms in drinking water distribution systems. Water Science and Technology: Water Supply 2, 271 – 280.

Flemming, H.-C., Wingender, J. (2002). Extracellular polymeric substances (EPS): structure, ecological and technical aspects. In: Encyclopedia of Environmental Microbiology, Vol. 3, pp. 1223 – 1231. Bitton, G. (Ed.), Wiley, New York.

Flemming, H.-C., Wingender, J. (2003). The crucial role of extracellular polymeric substances in biofilms. In: Biofilms in Wastewater Treatment: An Interdisciplinary Approach, pp. 178 – 210. Wuertz S., Bishop, P.L., Wilderer, P.A. (Eds.), IWA Publishing, London.

Flemming, H.-C., Neu, T. R., Wozniak, D. (2007). The EPS matrix: the „house of biofilm cells“. Journal of Bacteriology 189, 7945 – 7947.

Flemming, H.-C., Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology 8, 623 – 633.

Flemming, H.-C. (2011). Microbial Biofouling: Unsolved problems, insufficient approaches, and possible solutions. In: Biofilm Highlights. Springer Series on Biofilms, Vol. 5, pp. 81 – 109. Flemming, H.-C., Wingender, J., Szewzyk, U. (Eds.), Springer Heidelberg, Berlin.

Flemming, H.-C., Bendinger, B., Exner, M., Kistemann, T., Schaule, G., Szewzyk, U., Wingender, J. (2012). The last meters before the tap: where drinking water quality is at risk. In: Microbial growth in drinking water distribution systems and tap water installations. van der Kooij, D., van der Wielen, P. (Eds.), IWA Publishing, in press.

Fletcher, M., Loeb, G. I. (1979). Influence of substratum characteristics on the attachment of marine pseudomonad to solid surfaces. Applied and Environmental Microbiology 37, 67 – 72.

Forster, C. F., Clarke, A. R. (1983). The production of polymer from activated sludge by ethanolic extraction and its relation to treatment plant operation. Water Pollution Control 82, 430 – 433.

Forster, C. F., Quarmby, J. (1995). The physical characteristics of anaerobic granular sludges in relation to their internal architecture. Antonie van Leeuwenhoek 67, 103 – 110.

Frings, C. S., Fendley, T. W., Dunn, R. T., Queen, C. A. (1972). Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clinical Chemistry 18, 673 – 674.

Frølund, B., Griebe, T., Nielsen, P. H. (1995). Enzymatic activity in the activated-sludge floc matrix. Applied Microbiology and Biotechnology 43, 755 – 761.

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Research 30, 1749 – 1758.

Fronzes, R., Christie, P. J., Waksman, G. (2009). The structural biology of type IV secretion systems. Nature Reviews Microbiology 7, 703 – 714.

Fux, C. A., Costerton, J. W., Stewart, P. S., Stoodley, P. (2005). Survival strategies of infectious biofilms. Trends in Microbiology 13, 34 – 40.

Gaborieau, M., Castignolles, P. (2011). Size-exclusion chromatography (SEC) of branched polymers and polysaccharides. Analytical and Bioanalytical Chemistry 399, 1413 – 1423.

Gadd, G. M. (2000). Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Current Opinion in Biotechnology 11, 271 – 279.

Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156, 609 – 643.

Garber, N., Guempel, U., Belz, A., Gilboa-Garber, N., Doyle, R. J. (1992). On the specificity of the D-galactose-binding lectin (PA-1) of *Pseudomonas aeruginosa* and its strong binding to hydrophobic derivatives of D-galactose and thiogalactose. Biochimica et Biophysica Acta 1116, 331 – 333.

Garcia-Contreras, R., Zhang, X.-S., Kim, Y., Wood, T. K. (2008). Protein translation and cell death: the role of rare tRNAs in biofilm formation and in activating dormant phage killer genes. PLoS ONE 3, e2394.

Garfin, D., Heerdt, L. (2000). 2-D electrophoresis for proteomics: A methods and products manual. Bio-Rad bulletin 2651.

Gaudy, E., Wolfe, R. S. (1962). Composition of an extracellular polysaccharide produced by *Sphaerotilus natans*. Applied Microbiology 10, 200 – 205.

Geesey, G.G. (1982). Microbial exopolymers: ecological and economic considerations. ASM News 48, 9 – 14.

Gehr, R., Henry, J. G. (1983). Removal of extracellular material: techniques and pitfalls. Water Research 12, 1743 – 1748.

Gehrke, T., Telegdi, J., Thierry, D., Sand, W. (1998). Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Applied Environmental Microbiology 64, 2743 – 2747.

Gerdes, K., Christensen, S. K., Løbner-Olesen, A. (2005). Prokaryotic toxin-antitoxin stress response loci. Nature Reviews Microbiology 3, 371 – 382.

German Copper Institute (2010). Die fachgerechte Kupferrohr-Installation. Informationsdruck i.158. Überarbeitete Auflage.

Ghezzi, J. I., Steck, T. R. (1999). Induction of the viable but non-culturable condition in *Xanthomonas campestris* pv. *campestris* in liquid microcosms and sterile soil. FEMS Microbiology Ecology 30, 203 – 208.

Gião, M. S., Wilks, S. A., Azevedo, N. F., Vieira, M. J., Keevil, C. W. (2009). Comparison between standard culture and peptide nucleic acid 16S rRNA hybridization quantification to study the influence of physico-chemical parameters on *Legionella pneumophila* survival in drinking water biofilms. Biofouling 25, 343 – 351.

Gilbert, P., Collier, P. J., Brown, M. R. W. (1990). Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrobial Agents and Chemotherapy 34, 1865 – 1868.

Goel, R., Mino, T., Satoh, H., Matsuo, T. (1998). Comparison of hydrolytic enzyme systems in pure culture and activated sludge under different electron acceptor conditions. Water Science and Technology 37(4-5), 335 – 343.

Görg, A., Weiss, W., Dunn, M. J. (2004). Current two-dimensional electrophoresis technology for proteomics. Proteomics 4, 3665 – 3685.

Grey, B. E., Steck, T. R. (2001a). The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. Applied and Environmental Microbiology 67, 3866 – 3872.

Grey, B., Steck, T. R., (2001b). Concentrations of copper though to be toxic to *Escherichia coli* can induce the viable but nonculturable condition. Applied and Environmental Microbiology 67, 5325 – 5327.

Grossart, H. P., Steward, G. F., Martinez, J., Azam, F. (2000). A simple, rapid method for demonstrating bacterial flagella. Applied and Environmental Microbiology 66, 3632 – 3636.

Gruter, M., Leeflang, B. R., Kuiper, J., Kamerling, J. P., Vliegenthart, J. F. g. (1993). Structural characterisation of the exopolysaccharide produced by *Lactobacillus delbrückii* subspecies *bulgaricus* rr grown in skimmed milk. Carbohydrate research 239, 209 – 226.

Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2003). Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere 52, 1701 – 1710.

Guibaud, G., van Hullebusch, E., Bordas, F., d'Abzac, P., Joussein, E. (2009). Sorption of Cd(II) and Pb(II) by exopolymeric substances (EPS) extracted from activated sludges and pure bacterial strains: Modeling of the metal/ligand ratio effect and role of the mineral fraction. Bioresource Technology 100, 2959 – 2968.

Gutierrez-Gonzalvez, M. G., Armas-Portela, R., Stockert, J. C. (1987). Differential staining of biological structures by ruthenium red. Journal of Microscopy 145, 333 – 340.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology 17, 994 – 999.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Micobiology 2, 95 – 108.

Han, D. K., Eng, J., Zhou, H., Aebersold, R. (2001). Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. Nature Biotechnology 19, 946 – 951.

Hannig, C., Follo, M., Hellwig, E., Al-Ahmad, A. (2010). Visualization of adherent micro-organisms using different techniques. Journal of Medical Microbiology 59, 1 – 7.

Hansma, H. G., Pietrasanta, L. (1998). Atomic force microscopy and other scanning probe microscopies. Current Opinion in Chemical Biology 2, 579 – 584.

Harland, C. W., Botyanszki, Z., Rabuka, D., Bertozzi, C. R., Parthasarathy, R. (2009). Synthetic trehalose glycolipids confer desiccation resistance to supported lipid monolayers. Langmuir 25, 5193 – 5198.

Harris, R. F. (1981). Effect of water potential on microbial growth and activity. In: Water Potential Relations in Soil Microbiology. Soil Science Society of America Special Publication, Vol. 9, pp. 23 – 95. Parr., J. F., Gardner, W. R., Elliot, L. F. (Eds.), Soil Science Society of America, Madison.

Hausner, M., Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative *in situ* analysis. Applied and Environmental Microbiology 65, 3710 – 3713.

Hebbar, K. P., Gueniot, B., Heyraud, A., Colin-Morel, P., Heulin, T., Blandreau, J., Rinaudo, M. (1992). Characterization of exopolysaccharides produced by *Rhizobacteria*. Applied Microbiology and Biotechnology 38, 248 – 253.

Hejzlar, J., Chudoba, J. (1986). Microbial polymers in the aquatic environment - I: production by activated sludge microorganisms under different conditions. Water Research 20, 1209 – 1216.

Henrici, A. T. (1933). Studies of freshwater bacteria: I. A direct microscopic technique. Journal of Bacteriology 25, 277 – 287.

Higgins, M. J., Novak, J. T. (1997). Characterization of exocellular protein and its role in bioflocculation. Journal of Environmental Engineering 123, 479 – 485.

Hinsa, S. M., Espinosa-Urgel, M., Ramos, J. L., O'Toole, G. A. (2003). Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Molecular Microbiology 49, 905 – 918.

Hirose, I., Sano, K., Shioda, I., Kumano, M., Nakamura, K., Yamane, K. (2000). Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. Microbiology 146, 65 – 75.

Hoefel, D., Monis, P. T., Grooby, W. L., Andrews, S., Saint, C. P. (2005). Profiling bacterial survival through a water treatment process and subsequent distribution system. Journal of Applied Microbiology 99, 175 – 186.

Hoekstra, D., van der Laan, J. W., de Leij, L., and Witholt, B. (1976). Release of outer membrane fragments from normally growing *Escherichia coli*. Biochimica et Biophysica Acta 455, 889 – 899.

Holloway, C. F., Cowen, J. P. (1997). Development of a scanning confocal laser microscopic technique to examine the structure and composition of marine snow. Limnology and Oceanography 42, 1340 – 1352.

Hood, M. A., Schmidt, J. M. (1996). The examination of *Seliberia stellata* exopolymer using lectin assays. Microbial Ecology 31, 281 – 290.

Hoppe, H. G. (1991). Microbial extracellular enzyme activity: A new key parameter in aquatic ecology. In: Microbial Enzymes in Aquatic Environments, pp. 60 – 83. Chróst, R. J. (Ed.), Springer-Verlag, New York.

Horan, N. J., Eccles, C. R. (1986). Purification and characterization of extracellular polysaccharide from activated sludges. Water Research 20, 1427 – 1432.

Huang, Z., You, W., Haugland, R. P., Paragas, V. B., Olson, N. A., Haugland, R. P (1993). A novel fluorogenic substrate for detecting alkaline phosphatase activity *in situ*. Journal of Histochemistry and Cytochemistry 41, 313 – 317.

Huang, C.-T., Xu, K. D., McFeters, G. A., Stewart, P. S. (1998). Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. Applied and Environmental Microbiology 64, 1526 – 1531.

Huang, W. E., Ude, S., Spiers, A. J. (2007). *Pseudomonas fluorescens* SBW25 biofilm and planktonic cells have differentiable Raman spectral profiles. Microbial Ecology 53, 471 – 474.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiology and Molecular Biology Reviews 62, 379 – 433.

Ivleva, N. P., Wagner, M., Szkola, A., Horn, H., Niessner, R., Haisch, C. (2010). Label-free *in situ* SERS imaging of biofilms. The Journal of Physical Chemistry B 114, 10184 – 10194.

Jahn, A., Nielsen, P.H. (1995). Extraction of extracellular polymeric substances (EPS) from biofilms using a cation exchange resin. Water Science and Technology 32(8), 157 – 164.

Jann, B., Shashkov, A. S., Kochanowski, H., Jann, K. (1994). Structure of the O16 polysaccharide from *Escherichia coli* O16:K1: an NMR investigation. Carbohydrate Research 264, 305 – 311.

Jann, K., Jann, B., Schmidt, M. A., Vann, W. F. (1980). Structure of the *Escherichia coli* K2 capsular antigen, a teichoic acid-like polymer. Journal of Bacteriology 143, 1108 – 1115.

Jia, X. S., Furumai, H., Fang, H. H. P. (1996). Yields of biomass and extracellular polymers in four aerobic sludges. Environmental Technology 17, 283 – 291.

Jiao, Y., D'haeseleer, P., Dill, B. D., Shah, M., Verberkmoes, N. C., Hettich, R. L., Banfield, J. F., Thelen, M. P. (2011). Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. Applied and Environmental Microbiology 77, 5230 – 5237.

Johnsen, A. R., Hausner, M., Schnell, A., Wuertz, S. (2000). Evaluation of fluorescently labelled lectins for noninvasive localization of extracellular polymeric substances in *Sphingomonas* biofilms. Applied and Environmental Microbiology 66, 3487 – 3491.

Jones, S. E., Lock, M. A. (1989). Hydrolytic extracellular enzyme activity in heterotrophic biofilms from two contrasting streams. Freshwater Biology 22, 289 – 296.

Jones, S. E., Lock, M. A. (1991). Peptidase activity in river biofilms by product analysis. In: Mcrobial Enzymes in Aquatic Environments, pp 144 – 154. Chróst, R. J. (Ed.), Springer-Verlag, New York.

Jorand, F., Zartarian, F., Thomas, F., Block, J. C., Bottero, J. Y., Villemin, G., Urbain, V., Manem, J. (1995). Chemical and structural (2D) linkage between bacteria within activated sludge flocs. Water Research 29, 1639 – 1647.

Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Sharipo, A., Azevedo, N. F., Menard-Szczebara, F., Castagnet, S., Feliers, C., Keevil, C. W. (2007). Detection of *Escherichia coli* in biofilms from pipe samples and coupons in drinking water distribution networks. Applied and Environmental Microbiology 73, 7456 – 7464.

Junelles, A. M., Kanouni, A. E., Petitdemange, H., Gay, R. (1989). Influence of acetic and butyric acid addition on polysaccharide formation by *Clostridium acetobutylicum*. Journal of Industrial Microbiology 4, 121 – 125.

Kadurugamuwa, J. L., Mayer, A., Messner, P., Sara, M., Sleytr, U. B., and Beveridge, T. J. (1998). S-layered *Aneurinibacillus* and *Bacillus* spp. are susceptible to the lytic action of *Pseudomonas aeruginosa* membrane vesicles. Journal of Bacteriology 180, 2306 – 2311.

Kalmbach, S., Manz, W., Szewzyk, U. (1997). Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. FEMS Microbiology Ecology 22, 265 – 279.

Kang, D., Gho, Y. S., Suh, M., Kang, C. (2002). Highly sensitive and fast protein detection with Coomassie Brilliant Blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bulletin of the Korean Chemical Society 23, 1511 – 1512.

Kaplan, J. B., Ragunath, C., Ramasubbu, N., Fine, D. H. (2003). Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous ß-hexosaminidase activity. Journal of Bacteriology 185, 4693 – 4698.

Kaplan, J. B., Ragunath, C., Velliyagounder, K., Fine, D. H., Ramasubbu, N. (2004). Enzymatic detachment of *Staphylococcus epidermidis* biofilms. Antimicrobial Agents and Chemotherapy 48, 2633 – 2636.

Karapanagiotis, N. K., Rudd, T., Sterritt, R. M., Lester, J. N. (1989). Extraction and characterisation of extracellular polymers in digested sewage sludge. Journal of Chemical Technology and Biotechnology 44, 107 – 120.

Karatan, E., Watnick, P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiology and Molecular Biology Reviews 73, 310 – 347.

Karkhanis, Y. D., Zeltner, J. Y., Jackson, J. J., Carlo, D. J. (1978). A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. Analytical Biochemistry 85, 595 – 601.

Karlyshev, A. V., Wren, B. W. (2001). Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using Alcian blue dye. Journal of Clinical Microbiology 39, 279 – 284.

Karthikeyan, S., Wolfaardt, G. M., Korber, D. R., Caldwell, D. E. (1999). Functional and structural responses of a degradative microbial community to substrates with varying degrees of complexity in chemical structure. Microbial Ecology 38, 215 – 224.

Kawaguchi, T., Decho, A. W. (2000). Biochemical characterization of cyanobacterial extracellular polymers (EPS) from modern marine stromatolites (Bahamas). Preparative Biochemistry and Biotechnology 30, 321 – 330.

Keith, L. M., Bender, C. L. (1999). AlgT (σ^{22}) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. Journal of Bacteriology 181, 7176 – 7184.

Kennedy, A. F., Sutherland, I. W. (1987). Analysis of bacterial exopolysaccharides. Biotechnology and Applied Biochemistry 9, 12 – 19.

Kennedy, J. F., Palva, P. M. G., Corella, M. T. S., Cavalcanti, M. S. M., Coelho, L. C. B. B. (1995). Lectins, versatile proteins of recognition: a review. Carbohydrate Polymers 26, 219 – 230.

Kilb, B., Kuhlmann, B., Eschweiler, B., Preuß, G., Ziemann, E., Schöttler, U. (1998). Community structures of different groundwater habitats investigated using methods of molecular biology. Acta Hydrochimica et Hydrobiologia 26, 349 – 354.

Kilb, B., Lange, B., Schaule, G., Flemming, H.-C., Wingender, J. (2003). Contamination of drinking water by coliforms from biofilms grown on rubber-coated valves. International Journal of Hygiene and Environmental Health 206, 563 – 573.

Kim, C. W., Koopman, B., Bitton, G. (1994). INT-dehydrogenase activity test for assessing chlorine and hydrogen peroxide inhibition of filamentous pure cultures and activated sludge. Water Research 28, 1117 – 1121.

Kim, B. R., Andersson, J. E., Mueller, S. A., Gaines, W. A., Kendall, A. M. (2002). Literature review – efficacy of various disinfectants against *Legionella* in water systems. Water Research 36, 4433 – 4444.

Kim, E.-J., Wang, W., Deckwer, W.-D., Zeng, A.-P. (2005). Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of *Pseudomonas aeruginosa* irrespective of cell density. Microbiology 151, 1127 – 1138.

Kimura, S., Kondo, T. (2002). Recent progress in cellulose biosynthesis. Journal of Plant Research 115, 297 – 302.

King, R. O., Forster, C. F. (1990). Effects of sonication on activated sludge. Enzyme and Microbial Technology 12, 109 – 115.

Kistemann, T., Schreiber, C., Völker, S. (2010). Vorkommen mikrobieller Trinkwasserkontaminationen in Hausinstallationen. In: Vermeidung und Sanierung von Trinkwasser-Kontaminationen durch hygienisch relevante Mikroorganismen aus Biofilmen der Hausinstallation, Vol. 54, pp. 31 – 100. Flemming, H.-C. (Ed.), IWW Schriftenreihe Band 54, Mülheim.

Knirel, Y. A. (1990). Polysaccharide antigens of *Pseudomonas aeruginosa*. Critical Reviews in Microbiology 17, 273 – 304.

Krisch K. (1971). Carboxylic ester hydrolases. In: The Enzymes, Vol. 5, pp. 43 – 69. Boyer, P. D. (Ed.), Academic Press, London.

Korber, D. R., Caldwell, D. E., Costerton, J. W. (1994). Structural analysis of native and pure-culture biofilms using scanning confocal laser microscopy. In: Proceedings of the National Association of Corrosion Engineers (NACE), pp. 347 – 353. Canadian Region Western Conference, Calgary.

Körstgens, V., Flemming, H.-C., Wingender, J., Borchard, W. (2001). Influence of calcium ions on the mechanical properties of a model biofilm of mucoid *Pseudomonas aeruginosa*. Water Science and Technology 43(6), 49 – 57.

Kuehn, M. J., Kesty, N. C. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. Genes and Development 19, 2645 – 2655.

Kuhn, D. M., Chandra, J., Mukherjee, P. K., Ghannoum, M. A. (2002). Comparison of biofilms formed by Candida albicans and *Candida parapsilosis* on bioprosthetic surfaces. Infection and Immununity 70, 878 – 888.

Lamont, H. C., Silvester, W. B., Torrey, J. G. (1987). Nile red fluorescence demonstrates lipid in the envelope of vesicles from N2-fixing cultures of Frankia. Canadian Journal of Microbiology 34, 656 – 660.

Landeen, L. K., Yahya, M. T., Gerba, C. P. (1989). Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of Legionella pneumophila. Applied and Environmental Microbiology 55, 3045 – 3050.

Langille, S. E., Weiner, R. M. (1998). Spatial and temporal deposition of *Hyphomonas* strain VP-6 capsules involved in biofilm formation. Applied and Environmental Microbiology 64, 2906 – 2913.

Långmark, J., Storey, M. V., Ashbolt, N. J., Stenstrom, T. A. (2005). Biofilms in an urban water distribution system: measurement of biofilm biomass, pathogens and pathogen persistence within the Greater Stockholm Area, Sweden. Water Science and Technology 52(8), 181 – 189.

Lapeña, M. A., Pardo, C., Gacto, M. (1987). Effect of trehalose and other compounds on the resistance to desiccation by *Candida utilis* cells. Anales de Biologia 14, 25 – 31.

Larsen, P., Nielsen, J. L., Dueholm, M. S., Wetzel, R., Otzen, D., Nielsen, P. H. (2007). Amyloid adhesins are abundant in natural biofilms. Environmental Microbiology 9, 3077 – 3090.

Larsen, P., Nielsen, J. L., Otzen, D., Nielsen, P. H. (2008). Amyloid-like adhesins produced by floc-forming and filamentous bacteria in activated sludge. Applied and Environmental Microbiology 74, 1517 – 1526.

Lasa, I., Penadés, J. R. (2006). Bap, a family of surface proteins involved in biofilm formation. Research in Microbiology 157, 99 – 107.

Laspidou, C. S., Rittmann, B. E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 2711 – 2720.

Lattner, D., Flemming, H.-C., Mayer, C. (2003). ^{13}C-NMR study of the interaction of bacterial alginate with bivalent cations. International Journal of Biological Macromolecules 33, 81 – 88.

Laurent, P., Servais, P. (1995). Fixed bacterial biomass estimated by potential exoproteolytic activity. Canadian Journal of Microbiology 41, 749 – 752.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T., Hammes, F. (2010). Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. Water research 44, 4868 – 4877.

Lawrence, J. R., Neu, T. R., Swerhone, G. D. W. (1998a). Application of multiparameter imaging for the quantification of algal, bacterial and exopolymer components of microbial biofilms. Journal of Microbiological Methods 32, 253 – 261.

Lawrence, J. R., Wolfaardt, G. M., Neu, T. R. (1998b). The study of biofilms using confocal laser scanning microscopy. In: Digital analysis of microbes. Imaging, morphometry, fluorimetry and motility techniques and applications, pp. 431 – 465. Wilkinson, M. H. F., Schut, F. (Eds.), Modern microbiological methods series, Wiley, Sussex.

Lawrence, J. R., Neu, T. R. (2003). Microscale analyses of the formation and nature of microbial biofilm communities in river systems. Reviews in Environmental Science and Biotechnology 2, 85 – 97.

LeChevallier, M. W., Babcock, T. M., Lee, R. G. (1987). Examination and characterization of distribution system biofilms. Applied and Environmental Microbiology 53, 2714 – 2724.

LeChevallier, M. W., Norton, C. D., Camper, A., Morin, P., Ellis, B., Jones, W., Rompré, A., Prévost, M., Coallier, J., Servais, P., Holt, D., Delanoue, A., Colbourne, J. (1998). Microbial impact of biological filtration. American Water Works Association Research Foundation, USA.

Lee, D.-G., Lee, J.-H., Kim, S.-J. (2005). Diversity and dynamics of bacterial species in a biofilm at the end of the Seoul water distribution system. World Journal of Microbiology and Biotechnology 21, 155 – 162.

Leis, A., Flemming, H.-C. (2002). Carbon transformations and activity in biofilms. In: Encyclopedia of Environmental Microbiology, Vol. 1, pp. 81 – 92. Bitton, G. (Ed.), Wiley, New York.

Lehtola, M. J., Miettinen, I. T., Keinänen, M. M., Kekki, T. K., Laine, O., Hirvonen, A., Vartiainen, T., Martikainen, P. J. (2004). Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. Water Research 38, 3769 – 3779.

Lehtola, M. J., Torvinen, E., Kusnetsov, J., Pitkanen, T., Maunula, L., von Bonsdorff, C.-H., Martikainen, P. J., Wilks, S. A., Keevil, C. W., Miettinen, I. T. (2007). Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Applied and Environmental Microbiology 73, 2854 – 2859.

Lehman, R. M., O'Connell, S. P. (2002). Comparison of extracellular enzyme activities and community composition of attached and free-living bacteria in porous medium columns. Applied and Environmental Microbiology 68, 1569 – 1575.

Lemmer, H., Roth, D., Schade, M. (1994). Population density and enzyme activities of heterotrophic bacteria in sewer biofilms and activated sludge. Water Research 28, 1341 – 1346.

Leriche, V., Sibille, P., Carpentier, B. (2000). Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. Applied and Environmental Microbiology 66, 1851 – 1856.

Lewandowski, Z., Altobelli, S. A., Majors, P. D., Fukushima, E. (1992). NMR imaging of hydrodynamics near microbially colonized surfaces. Water Science and Technology 26(3-4), 577 – 584.

Lewandowski, Z., Stoodley, P., Altobelli, S. (1995). Experimental and conceptual studies on mass transport in biofilms. Water Science and Technology 31(1), 153 – 162.

Li, X. , Logan, B. E. (2004). Analysis of bacterial adhesion using a gradient force analysis and colloid probe atomic force microscopy . Langmuir 20, 8817 – 8822.

Liu, H., Fang, H. P. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. Journal of Biotechnology 95, 249 – 256.

Liu, H., Fang, H. P. (2003). Influences of extracellular polymeric substances (EPS) on flocculation, settling and dewatering of activated sludge. Critical Reviews in Environmental Science and Technology 33, 237 – 273.

Loewenthal, R. E., Morrison, I., Wentzel, M. C. (2004). Control of corrosion and aggression in drinking water systems. Water Science and Technology 49(2), 9 – 18.

Logan, S. M., Trust, T. J. (1982). Outer membrane characteristics of *Campylobacter jejuni*. Infection and Immunity 38, 898 – 906.

Lorenz, M. G., Gerjets, D., Wackernagel, W. (1991). Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. Archives of Microbiology 156, 319 – 326.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265 – 275.

Madsen, J. S., Burmølle, M., Hansen, L. H., Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunology and Medical Microbiology 65, 183 – 195.

Majors, P. D., McLean, J. S., Pinchuk, G. E., Fredrickson, J. K., Gorby, Y. A., Minard, K. R., Wind, R. A. (2005). NMR methods for *in situ* biofilm metabolism studies. Journal of Microbiological Methods 62, 337 – 344.

Mallet, C., Debroas, D. (2001). Regulation of ß- and α-glycolytic activities in the sediments of a eutrophic lake. Microbial Ecology 41, 106 – 113.

Manca, M. C., Lama, L., Improta, R., Esposito, E., Gambacorta, A., Nicolaus, B. (1996). Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. Applied and Environmental Microbiology 62, 3265 – 3269.

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Systematic and Applied Microbiology 15, 593 – 600.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K. H., Stenstron, T. A. (1993). In-situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S ribosomal RNA directed and 23S ribosomal RNA directed fluorescent oligonucleotide probes. Applied Environmental Microbiology 59, 2293 – 2298.

Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Stenstrom, T. A., Hutzler, P., Schleifer, K. H. (1995). *In situ* identification of *Legionellaceae* using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. Microbiology 141, 29 – 39.

Manz, W., Wendt-Potthoff, K., Neu, T. R., Szewzyk, U., Lawrence, J. R. (1999). Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy. Microbial Ecology 37, 225 – 237.

Manz, B., Volke, F., Goll, D., Horn, H. (2003). Measuring local flow velocities and biofilm structure in biofilm systems with magnetic resonance imaging (MRI). Biotechnology and Bioengineering 84, 424 – 432.

Marshall, K. C. (1992). Biofilms: an overview of bacterial adhesion, activity and control at surfaces. ASM News 58, 202 – 207.

Martiny, A. C., Jorgensen, T. M., Albrechtsen, H.-J., Arvin, E., Molin, S. (2003). Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system. Applied and Environmental Microbiology 69, 6899 – 6907.

Mathieu, L., Bouteleux, C., Fass, S., Angel, E., Block, J. C. (2009). Reversible shift in the alpha-, beta- and gamma-proteobacteria populations of drinking water biofilms during discontinuous chlorination. Water Research 43, 3375 – 3386.

Matsushita, K., Adachi, O., Shinagawa, E., Ameyama, M. (1978). Isolation and characterization of outer and inner membranes from *Pseudomonas aeruginosa* and effect of EDTA on the membranes. Journal of Biochemistry 83, 171 – 181.

Mattila, K., Carpen, L., Hakkarainen, T., Salkinoja-Salonen, M. S. (1997). Biofilm development during ennoblement of stainless steel in baltic sea water: a microscopic study. International Biodeterioration and Biodegradation 40, 1 – 10.

May, T. B., Chakrabarty, A. M. (1994). Isolation and assay of *Pseudomonas aeruginosa* alginate. Methods in Enzymology 235, 295 – 304.

Mayansky, A. N., Chebotar, I. V., Rudneva, E. I., Chistyakova, V. P. (2012). *Pseudomonas aeruginosa*: characteristics of the biofilm process. Molecular Genetics, Microbiology and Virology 27, 1 – 6.

Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J., Flemming, H.-C. (1999). The role of intermolecular interactions studies on model systems for bacterial biofilms. International Journal of Biological Macromolecules 26, 3 – 16.

Mayer, C., Lattner, D., Schürks, N. (2001). ^{13}C NMR studies on selectively labeled bacterial biofilms. Journal of Industrial Microbiology and Biotechnology 26, 62 – 69.

McDougald, D., Rice, S. A., Barraud, N., Steinberg, P. D., Kjelleberg, S. (2012). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nature Reviews Microbiology 28, 39 – 50.

Measures, J. C. (1975). Role of amino acids in osmoregulation of non-halophilic bacteria. Nature 257, 398 – 400.

Meisen, S., Wingender, J., Telgheder, U. (2008). Analysis of microbial extracellular polysaccharides in biofilms by HPLC. Part I: Development of the analytical method using two complementary stationary phases. Analytical and Bioanalytical Chemistry 391, 993 – 1002.

Mikolay, A., Huggett, S., Tikana, L., Grass, G., Braun, J., Nies, D. H. (2010). Survival of bacteria on metallic copper surfaces in a hospital trial. Applied Microbiology and Biotechnology 87, 1875 – 1879.

Miksch, K. (1985). Auswahl einer optimalen Methodik für die Aktivitätsbestimmung des Belebtschlammes mit Hilfe des TTC-Test. Vom Wasser 64, 187 – 198.

Mistry, K., Krull, I., Grinberg, N. (2003). Size-exclusion capillary electrochromatographic separation of polysaccharides using polymeric stationary phases. Electrophoresis 24, 1753 – 1763.

Molecular Probes (2008). Handbook of fluorescent probes and research chemicals. 11th edition. Eugene, OR: Molecular Probes.

Molin, S., Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Current Oppinion in Biotechnology 14, 255 – 261.

Morgan, J. W., Forster, C. F., Evison, L. (1990). A comparative study of the nature of biopolymers extracted from anaerobic and activated sludges. Water Research 24, 743 – 750.

Moritz, M. M., Flemming, H.-C., Wingender, J. (2010). Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. International Journal of Hygiene and Environmental Health 213, 190 – 197.

Moritz, M. M. (2011). Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials. Dissertation, University of Duisburg-Essen, Germany.

Morse, E. E. (1947). Anthrone in estimating low concentrations of sucrose. Analytical Chemistry 19, 1012 – 1013.

Murga, R., Forster, T. S., Brown, E., Pruckler, J. M., Fields, B. S., Donlan RM (2001). Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147, 3121 – 3126.

Murray, A. E., Hollibaugh, J. T., Orrego, C. (1996). Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Applied and Environmental Microbiology 62, 2676 – 2680.

Muto, Y., Goto, S. (1986). Transformation by extracellular DNA produced by *Pseudomonas aeruginosa*. Microbiology and Immunology 30, 621 – 628.

Muyzer, G., de Waal, E. C., Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695 – 700.

Nandakumar, M. P., Cheung, A., Marten, M. R. (2006). Proteomic analysis of extracellular proteins from *Escherichia coli* W3110. Journal of Proteome Research 5, 1155 – 1161.

Naito, T., Kusano, K., Kobayashi, I. (1995). Selfish behavior of restriction-modification systems. Science 267, 897 – 899.

Nelson, R., Sawaya, M. R., Balbirnie, M., Madsen, A. Ø., Riekel, C., Grothe, R., Eisenberg, D. (2005). Structure of the cross-β spine of amyloid-like fibrils. Nature 435, 773 – 778.

Neu, T. R., Marshall, K. C. (1991). Microbial "footprints" – a new approach to adhesive polymers. Biofouling 3, 101 – 112.

Neu, T. R., Lawrence, J. R. (1997). Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. FEMS Microbiology Ecology 24, 11 – 25.

Neu, T. R., Lawrence, J. R. (1999). Lectin-binding analysis in biofilm systems. Methods in Enzymology 310, 145 – 152.

Neu, T. R., Swerhone, G. D. W., Lawrence, J. R. (2001). Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. Microbiology 147, 299 – 313.

Neu, T. R., Swerhone, G. D. W., Böckelmann, U., Lawrence, J. R. (2005). Effect of CNP on composition and structure of lotic biofilms as detected with lectin-specific glycoconjugates. Aquatic Microbial Ecology 38, 283 – 294.

Neurath, A. R. (1966). Interference of sodium ethylenediaminetetraacetate in the determination of proteins and its elimination. Experientia 22, 290.

Ng, F. M.-W., Dawes, E. A. (1973). Chemostat studies on the regulation of glucose metabolism in *Pseudomonas aeruginosa* by citrate. Biochememical Journal 132, 129 – 140

Nielsen, P. H., Frølund, B., Spring, S., Caccavo, F. (1997). Microbial Fe(III) reduction in activated sludge. Systematic and Applied Microbiology 20, 645 – 651.

Nielsen, P. H., Keiding, K. (1998). Disintegration of activated sludge flocs in presence of sulfide. Water Research 32, 313 – 320.

Nielsen, P. H., Jahn, A. (1999). Extraction of EPS. In: Microbial extracellular polymeric substances, pp. 49 – 72. Wingender, J., Neu, T.R., Flemming, H.-C. (Eds.), Springer Verlag, Berlin.

Nielsen, A. T., Tolker-Nielsen, T., Barken, K. B., Molin, S. (2000). Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. Environmental Microbiology 2, 59 – 68.

Nies, D. H., Silver, S. (1995). Ion efflux systems involved in bacterial metal resistances. Journal of Industrial Microbiology and Biotechnology 14, 186 – 199.

Nishikawa, S., Kuriyama, M. (1968). Nucleic acid as a component of mucilage in activated sludge. Water Research 2, 811 – 812.

Nithya, C., Begum, M. F., Pandian, S. K. (2010). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. Applied Microbiology and Biotechnology 88, 341 – 358.

Nivens, D. E., Chambers, J. Q., Anderson, T. R., White, D. C. (1993). Long-term, on-line monitoring of microbial biofilms using a quartz crystal microbalance. Analytical Chemistry 65, 65 – 69.

Nocker, A., Sossa-Fernandez, P., Burr, M. D., Camper, A. K. (2007). Use of propidium monoazide for live/dead distinction in microbial ecology. Applied and Environmental Microbiology 73, 5111 – 5117.

Nouwens, A. S., Beatson, S. A., Whitchurch, C. B., Walsh, B. J., Schweizer, H. P., Mattick, J. S., Cordwell, S. J. (2003). Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology 149, 1311 – 1322.

Noyce, J. O., Michels, H., Keevil, C. W. (2006). Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant Staphylococcus aureus in the healthcare environment. The Journal of hospital infection 63, 289 – 297.

Nybroe, O., Jørgensen, P. E., Henze, M. (1992). Enzyme activities in wastewater and activated sludge. Water Research 26, 579 – 584.

O'Connell, D. (2007). Biofilms to die for… . Nature Reviews Microbiology 5, 398.

O'Ferrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. The Journal of Biological Chemistry 250, 4007 – 4021.

O'Toole, G., Kaplan, H.B., Kolter, R. (2000). Biofilm formation as microbial development. Annual Review of Microbiology 54, 49 – 79.

Okabe, S., Satoh, H., Watanabe, Y. (1999). *In situ* analysis of nitrifying biofilms as determined by *in situ* hybridization and the use of microelectrodes. Applied and Environmental Microbiology 65, 3182 – 3191.

Okabe, S., Kindaichi, T., Ito, T. (2005). Fate of ^{14}C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Applied and Environmental Microbiology 71, 3987 – 3994.

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. Journal of Microbiology 43 spec. issue, 93 – 100.

Omar, A. S., Weckesser, J., Mayer, H. (1983). Different polysaccharides in the external layers (capsule and slime) of the cell envelope of *Rhodopseudomonas capsulata* Sp11. Archives of Microbiology 136, 291 – 296.

Omoike, A., Chorover, J. (2004). Spectroscopic study of extracellular polymeric substances from *Bacillus subtilis*: aqueous chemistry and adsorption effects. Biomacromolecules 5, 1219 – 1230.

Ordax, M., Marco-Noales, E., López, M. M., Biosca, E. G. (2006). Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. Applied and Environmental Microbiology 72, 3482 – 3488.

Palmgren, R., Nielsen, P.H. (1996). Accumulation of DNA in the exopolymeric matrix of activated sludge and bacterial cultures. Water Science and Technology 34(5-6), 233 – 240.

Pamp, S. J., Gjermansen, M., Tolker-Nielsen, T. (2007). The biofilm matrix: a sticky framework. In: The Biofilm Mode of Life: Mechanisms and Adaptations, pp. 37 – 69. Kjelleberg, S. and Givskov, M. (Eds.), Horizon Scientific Press, London.

Panchaud, A., Hansson, J., Affolter, M., Bel Rhlid, R., Piu, S., Moreillon, P., Kussmann, M. (2008). ANIBAL, stable isotope-based quantitative proteomics by aniline and benzoic acid labeling of amino and carboxylic groups. Molecular and Cellular Proteomics 7, 800 – 812.

Pandey, D. P., Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Research 33, 966 – 976.

Pantke, M. (1996). General aspects and test methods. In: Microbially Infiuenced Corrosion of Materials - Scientfic and Technological Aspects, pp. 379 – 392. E. Heitz, W. Sand, H.-C. Flemming (Eds.), Springer, Berlin.

Park, C., Novak, J. T. (2007). Characterization of activated sludge exocellular polymers using several cation-associated extraction methods. Water Research 41, 1679 – 1688.

Paul, J. H., Jeffrey, W. H. (1985). Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*. Applied and Environmental Microbiology 50, 431 – 437.

Pavissich, J. P., Vargas, I. T., González, B., Pastén, P. A., Pizarro, G. E. (2010). Culture dependent and independent analyses of bacterial communities involved in copper plumbing corrosion. Journal of Applied Microbiology 109, 771 – 782.

Pavoni, J. L., Tenney, M. W., Echelberger, W. F. (1972). Bacterial exocellular polymers and biological flocculation. Journal of the Water Pollution Control Federation 44, 414 – 431.

Pedersen, K. (1990). Biofilm development on stainless steel and PVC surfaces in drinking water. Water Research 24, 239 – 243.

Pelkonen, S., Häyrinen, J., Finne, J. (1988). Polyacrylamide gel electrophoresis of capsular polysaccharides of *Escherichia coli* and other bacteria. Journal of Bacteriology 170, 2646 – 2653.

Pepper, I. L., Rusin, P., Quintanar, D. R., Haney, C., Josephson, K. L., Gerba, C. P. (2004). Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. International Journal of Food Microbiology 92, 289 – 295.

Percival, S. L., Knapp, J. S., Wales, D. S., Edyvean, R. G. J. (1999). The effect of turbulent flow and surface roughness on biofilm formation in drinking water. Journal of Industrial Microbiology and Biotechnology 22, 152 – 159.

Perkins, D. N., Pappin, D. J. C., Creasy, D. M., Cottrell, J., S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551 – 3567.

Peterson, G. L. (1977). A simplification of the protein assay method of lowry *et al.* which is more generally applicable. Analytical Biochemistry 83, 346 – 356.

Peterson, G. L. (1979). Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Analytical Biochemistry 100, 201 – 220.

Petersen, F. C., Pecharki, D., Scheie, A. A. (2004). Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. Journal of Bacteriology 186, 6327 – 6331.

Pierre, G., Graber, M., Rafiliposon, B. A., Dupuy, C., Orvain, F., De Crignis, M., Maugard, T. (2012). Biochemical composition and changes of extracellular polysaccharides (ECPS) produced during microphytobenthic biofilm development (Marennes-Oléron, France). Microbial Ecology 63, 157 – 169.

Prévost, M., Rompré, A., Coallier, J., Servais, P., Laurent, P., Clément, B., Lafrance, P. (1998). Suspended bacterial biomass and activity in full-scale drinking water distribution systems: impact of water treatment. Water Research 32, 1393 – 1406.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M. T., Guerri, G., Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. Biology and Fertility of Soils 45, 219 – 235.

Pinchuk, G. E., Ammons, C., Culley, D. E., Li, S.-M. W., McLean, J. S., Romine, M. F., Nealson, K. H., Fredrickson, J. K., Beliaev, A. S. (2008). Utilization of eDNA as a sole source of phosphorus, carbon and energy by *Shewanella* spp.: Ecological and physiological implications for dissimilatory metal reduction. Applied Environmental Microbiology 74, 1198 – 1208.

Platt, R.M., Geesey, G.G., Davis, J.D., White, D.C. (1985). Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. Canadian Journal of Microbiology 31, 675 – 680.

Potts, M. (1994). Desiccation tolerance of prokaryotes. Microbiology Reviews 58, 755 – 805.

Pozos, N., Scow, K., Wuertz, S., Darby, J. (2004). UV disinfection in a model distribution system: biofilm growth and microbial community. Water Research 38, 3083 – 3091.

Priester, J. H., Horst, A. M., van de Werfhorst, L. C., Saleta, J. L., Mertes, L. A. K., Holden, P. A. (2007). Enhanced visualization of microbial biofilms by staining and environmental scanning electron microscopy. Journal of Microbiological Methods 68, 577 – 587.

Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S., Qu, D. (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology 153, 2083 – 2092.

Quarmby, J., Forster, C. F. (1995). An examination of the structure of UASB granules. Water Research 11, 2449 – 2454.

Quintero, E. J., Weiner, R. M. (1995). Evidence for the adhesive function of the exopolysaccharide of *Hyphomonas* strain MHS-3 in its attachment to surface. Applied and Environmental Microbiology 61, 1897 – 1903.

Rahman, M. M., Guard-Petter, J., Asokan, K., Carlson, R. W. (1997). The structure of the capsular polysaccharide from a swarming strain of pathogenic *Proteus vulgaris*. Carbohydrate Research 301, 213 – 220.

Ram, R. J., Verberkmoes, N. C., Thelen, M. P., Tyson, G. W., Baker, B. J., Blake II, R. C., Shah, M., Hettich, R. L., Banfield, J. F. (2005). Community proteomics of a natural microbial biofilm. Science 308, 1915 – 1920.

Ramaswamy, S., Dworkin, M., Downard, J. (1997). Identification and characterization of *Myxococcus xanthus* mutants deficient in Calcofluor White binding. Journal of Bacteriology 179, 2863 – 2871.

Ras, M., Girbal-Neuhauser, E., Paul, E. M., Sperandio, M., Lefebvre, D. (2008). Protein extraction from activated sludge: An analytical approach. Water Research 42, 1867 – 1878.

Rättö, M., Verhoef, R., Suihko, M.-L., Blanco, A., Schols, H. A., Voragen, A. G. J., Wilting, R., Siika-Aho, M., Buchert, J. (2006). Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes. Journal of Industrial Microbiology and Biotechnology 33, 359 – 367.

Read, R. R., Costerton, J. W. (1987). Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. Canadian Journal of Microbiology 33, 1080 – 1090.

Rémy, A., Imam-Sghiouar, N., Poirier, F., Joubert-Caron, R. (2000). Focusing strategy and influence of conductivity on isoelectric focusing in immobilized pH gradients. Bio-Rad bulletin 2778.

Rendueles, O., Ghigo, J.-M. (2012). Multi-species biofilms: how to avoid unfriendly neighbors. FEMS Microbiology Reviews 36, 972 – 989.

Rice, K. C., Mann, E. E., Endres, J. L., Weiss, E. C., Cassat, J. E., Smeltzer, M. S., Bayles, K. W. (2007). The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proceedings of the National Academy of Sciences 104, 8113 – 8118.

Rice, S. A., Tan, C. H., Mikkelsen, P. J., Kung, V., Woo, J., Tay, M., Hauser, A., McDougald, D., Webb, J. S., Kjelleberg, S. (2009). The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. ISME Journal 3, 271 – 282.

Richards, S. R., Turner, R. J. (1984). A comparative study of techniques for the examination of biofilms by scanning electron microscopy. Water Research 18, 767 – 773.

Riemann, L., Steward, G. F., Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. Applied and Environmental Microbiology 66, 578 – 587.

Rippe, K., Guthold, M., von Hippel, P. H., Bustamante, C. (1997). Transcriptional activation via DNA-looping: visualization of intermediates in the activation pathway of *E. coli* RNA polymerase σ^{54} holoenzyme by scanning force microscopy. Journal of Molecular Biology 270, 125 – 138.

Roberson, E., Firestone, M. (1992). Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. Applied and Environmental Microbiology 58, 1284 – 1291.

Rode, A. (2004). Isolierung und Charakterisierung von bakteriellen extrazellulären polymeren Substanzen aus Biofilmen. Dissertation, Universty of Duisburg-Essen, Germany.

Rodriguez, G. G., Phipps, D., Ishiguro, K., Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Applied Environmental Microbiology 58, 1801 – 1808.

Roeder, R. S., Heeg, K., Tarne, P., Benölken, J. K., Schaule, G., Bendinger, B., Flemming, H.-C., Szewzyk, U. (2010a). Influence of materials, water qualities and disinfection methods on the drinking water biofilm community. Water Practice and Technology 5, published online.

Roeder, R. S., Lenz, J., Tarne, P., Gebel, J., Exner, M., Szewzyk, U. (2010b). Long-term effects of disinfectants on the community composition of drinking water biofilms. International Journal of Hygiene and Environmental Health 213, 183 – 189.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V., Keevil, C. W. (1994). Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems. Applied and Environmental Environmental Microbiology 60, 1842 – 1851.

Romani, A. M., Sabeter, S. (1999). Effect of primary producers on the heterotrophic metabolism of a stream biofilm 41, 729 – 736.

Romani, A. M., Fund, K., Artigas, J., Schwartz, T., Sabater, S., Obst, U. (2008). Relevance of polymeric matrix enzymes during biofilm formation. Microbial Ecology 56, 427 – 436.

Romero, D., Aguilar, C., Losick, R., Kolter, R. (2009). Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. Proceedings of the National Academy of Sciences 107, 2230 – 2234.

Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. Archives of Biochemistry and Biophysics 67, 10 – 15.

Rosenzweig, R., Shavit, U., Furman, A. (2012). Water retention curves of biofilm-affected soils using xanthan as an analogue. Soil Science Society of America Journal 76, 61 – 69.

Ross, N. W., Levitan, R., Labelle, J., Schneider, H. (1991). Protein and other compositional differences of the extracellular material from slimy and non-slimy colonies of non-mucoid *Pseudomonas aeruginosa*. FEMS Microbiology Letters 81, 257 – 260.

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bertlet-Joens, M., He, F., Jacobson, A., Pappin, D. J. (2004). Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Molecular and Cellular Proteomics 3, 1154 – 1169.

Ross, P., Mayer, R., Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. Microbiological Reviews 55, 35 – 58.

Rudd, T., Sterritt, R. M., Lester, J. (1982). The use of extraction methods for the quantification of extracellular polymer production by *Klebsiella aerogenes* under varying cultural conditions. Applied Microbiology and Biotechnology 16, 23 – 27.

Rudd, T., Sterritt, R. M., Lester, J. N. (1983). Extraction of extracellular polymers from activated sludge. Biotechnology Letters 5, 327 – 332.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. Journal of Molecular Biology 279, 485 – 499.

Ryssov-Nielsen, H. (1975). Measurement of the inhibition of respiration in activated sludge by a modified determination of the TTC-dehydrogenase activity. Water Research 9, 1179 – 1185.

Samek, O., Al-Marashi, J. F. M., Telle, H. H. (2010). The potential of Raman spectroscopy for the identification of biofilm formation by *Staphylococcus epidermidis*. Laser Physics Letters 7, 378 – 383.

Sand, W., Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. Research in Microbiology 157, 49 – 56.

Sanford, B. A., Thomas, V. L., Mattingly, S. J., Ramsay, M. A., Miller, M. M. (1995). Lectin-biotin assay for slime present in *in situ* biofilm produced by *Staphylococcus epidermidis* using transmission electron microscopy (TEM). Journal of Industrial Microbiology 15, 156 – 161.

Sato, T., Ose, Y. (1984). Floc-forming substances extracted from activated sludge with ammonium hydroxide and EDTA solutions. Water Science and Technology 17(4-5), 517 – 528.

Sauer, K., Camper, A. K. (2001). Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. Journal of Bacteriology 183, 6579 – 6589.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., Davies, D. G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology 184, 1140 – 1154.

Schauer, C., Hasselwander, H., Minar, A. (2008). Erfolgreiche Sanierung und Desinfektion einer komplexen Trinkwasseranlage. Energie Wasser-Praxis 4, 30 – 37.

Schaule, G., Flemming, H.-C., Ridgway, H. F. (1993b). The use of CTC in the quantification of respiratory active bacteria in biofilms. Applied and Environmental Microbiology 59, 3850 – 3857.

Schmeisser, C., Stöckigt, C., Raasch, C., Wingender, J., Timmis, K. N., Wenderoth, D. F., Flemming, H.-C., Liesegang, H., Schmitz, R. A., Jaeger, K.-E., Streit, W. R. (2003). Metagenome survey of biofilms in drinking-water networks. Applied and Environmental Microbiology 69, 7298 – 7309.

Schmidt, M. A., Jann, K. (1982). Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extraintestinal infections. FEMS Microbiology Letters 14, 69 – 74.

Schmidt, J. E., Ahring, B. K. (1994). Extracellular polymers in granular sludge from different upflow anaerobic sludge blanket (UASB) reactors. Applied Microbiology and Biotechnology 42, 457 – 462.

Schmitt, J., Nivens, D., White, D. C., Flemming, H. (1995). Changes of biofilm properties in response to sorbed substances - an FTIR-ATR study. Water Science and Technology 32(8), 149 – 155.

Schneider, T., Riedel, K. (2010). Environmental proteomics: analysis of structure and function of microbial communities. Proteomics 10, 785 – 798.

Schooling, S. R., Beveridge, T. J. (2006). Membrane vesicles: an overlooked component of the matrices of biofilms. Journal of Bacteriology 188, 5945 – 5947.

Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R., Schleifer, K. H. (1996). Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. Applied and Environmental Microbiology 62, 4641 – 4647.

Schürks, N., Wingender, J., Flemming, H.-C., Mayer, C. (2002). Monomer composition and sequence of alginates from *Pseudomonas aeruginosa*. International journal of biological macromolecules 30, 105 – 111.

Schwartz, T., Hoffmann, S., Obst, U. (2003). Formation of natural biofilms during chlorine dioxide and u.v. disinfection in a public drinking water distribution system. Journal of Applied Microbiology 95, 591 – 601.

Sesay, M. L., Özcengiz, G., Sanin, F. D. (2006). Enzymatic extraction of activated sludge extracellular polymers and implications on bioflocculation. Water Research 40, 1359 – 1366.

Seymour, J. D., Codd, S. L., Gjersing, E. L., Stewart, P. S. (2004). Magnetic resonance microscopy of biofilm structure and impact on transport in a capillary bioreactor. Journal of Magnetic Resonance 167, 322 – 327.

Sheng, G.-P., Yu, H.-Q., Yu, Z. (2005). Extraction of extracellular polymeric substances from the photosynthetic bacterium *Rhodopseudomonas acidophila*. Applied Microbiology and Biotechnology 67, 125 – 130.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances 28, 882 – 894.

Sillankorva, S., Neubauer, P., Azeredo, J. (2010). Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. Biofouling 26, 567 – 575.

Silver, S. (1996). Bacterial resistances to toxic metal ions – a review. Gene 179, 9 – 19.

Singer, V. L., Jones, L. J., Yue, S. T., Haugland, R. P. (1997). Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. Analytical Biochemistry 249, 228 – 238.

Sinsabaugh, R. L., Repert, D., Weiland, T., Golladay, S. W., Linkins, A. E. (1991). Exoenzyme accumulation in epilithic biofilms. Hydrobiologia 222, 29 – 37.

Sizemore, R. K., Caldwell, J. J., Kendrick A. S. (1990). Alternate Gram staining technique using a fluorescent lectin. Applied and Environmental Microbiology 56, 2245 – 2247.

Skraber, S., Schijven, J., Gantzer, C., de Roda Husman, A. M. (2005). Pathogenic viruses in drinking-water biofilms: a public health risk. Biofouling 2, 105 – 117.

Smith, D. C., Simon, M., Alldredge, A. L., Azam, F. (1992). Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature 359, 139 – 142.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76 – 85.

Smith, C. J., Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiology Ecology 67, 6 – 20.

Smithies, W. R., Gibbons, N. E. (1955). The deoxyribose nucleic acid slime layer of some halophilic bacteria. Canadian Journal of Microbiology 1, 614 – 621.

Smucker, R. A., Kim, C. K. (1991). Microbial extracellular enzyme activity: a new key parameter in aquatic ecology. In: Microbial enzymes in aquatic environments, pp. 60 – 83. R. J. Chrost (Ed.), Springer-Verlag, New York.

Späth, R., Flemming, H.-C., Wuertz, S. (1998). Sorption properties of biofilms. Water Science and Technology 37(4-5), 207 – 210.

Steele, A., Goddard, D. T., Beech, I. B. (1994). An atomic force microscopy study of the biodeterioration of stainless steel in the presence of bacterial biofilms. International Biodeterioration and Biodegradation 34, 35 – 46.

Steiner, A. E., McLaren, D. A., Forster, C. F. (1976). The nature of activated sludge flocs. Water Research 10, 25 – 30.

Stoeckigt, C., Schmeisser, C., Streit, W. R. (2003). Skimming the metagenome of a drinking water biofilm. Unpublished.

Stocks, S. M. (2004). Mechanism and use of the commercially available viability stain, *Bac*Light. Cytometry Part A 61A, 189 – 195.

Stoodley, P., de Beer, D., Lewandowski, Z. (1994). Liquid flow in biofilm systems. Applied and Environmental Microbiology 60, 2711 – 2716.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W. (2002). Biofilms as complex differentiated communities. Annual Review of Microbiology 56, 187 – 209.

Strathmann, M., Wingender, J., Flemming, H.-C. (2002) Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa.* Journal of Microbiological Methods 50, 237 – 248.

Straub, T. M., Gerba, C. P., Zhou, X., Price, R., Yahys, M. T. (1995). Synergistic inactivation of *E. coli* and MS-2 coliphage by chloramine and cupric chloride. Water Research 29, 811 – 818.

Suci, P. A., Mittelman, M. W., Yu, F. P., Geesey, G. G. (1994). Investigation of ciprofloxacin penetration into *Pseudomonas aeruginoas* biofilms. Antimicrobial Agents and Chemotherapy 38, 2125 – 2133.

Suci, P. A., Geesey, G. G., Tyler, B. J. (2001). Integration of Raman microscopy, differential interference contrast microscopy, and attenuated total reflection Fourier transform infrared spectroscopy to investigate chlorhexidine spatial and temporal distribution in *Candida albicans* biofilms. Journal of Microbiological Methods 46, 193 – 208.

Surman, S. B., Walker, J. T., Goddard, D. T., Morton, L. H. G., Keevil, C. W., Weaver, W., Skinner, A., Hanson, K., Caldwell, D., Kurtz, J. (1996). Comparison of microscope techniques for the examination of biofilms. Journal of Microbiological Methods 25, 57 – 70.

Sutherland, I.W. (1988). Bacterial surface polysaccharides: structure and function. International Review of Cytology 113, 187 – 231.

Sutherland, I.W. (1990). Exopolysaccharide structure. In: Biotechnology of microbial exopolysaccharides, Vol. 9, pp. 20 – 37. Sutherland, I.W. (Ed.), Cambridge University Press, Cambridge.

Sutherland, I.W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147, 3 – 9.

Szewzyk, U., Szewzyk, R., Manz, W., Schleifer, K. H. (2000). Microbiological safety of drinking water. Annual Review of Microbiology 54, 81 – 127.

Szewzyk, U., Szewzyk, R. (2003). Biofilms – die etwas andere Lebensweise. BIOspectrum 9, 253 – 255.

Tago, Y., Aida, K. (1977). Exocellular mucopolysaccharide closely related to bacterial floc formation. Applied and Environmental Microbiology 34, 306 – 314.

Tapia, J. M., Muñoz, J. A., González, F., Blázquez, M. L., Malki, M., Ballester, A. (2009). Extraction of extracellular polymeric substances from the acidophilic bacterium *Acidiphilium* 3.2Sup(5). Water Science and Technology 59(10), 1959 – 1967.

Taylor Eighmy, T., Bishop, P. L. (1985). Effect of reactor turbulence on the binding-protein-mediated aspartate transport system in thin wastewater biofilms. Applied and Environmental Microbiology 50, 120 – 124.

Teitzel, G. M., Parsek, M. K. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 69, 2313 – 2320.

Teitzel, G. M., Geddie, A., De Long, S. K., Kirisits, M. J., Whiteley, M., Parsek, M. R. (2006). Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. Journal of Bacteriology 188, 7242 – 7256.

Teng, F., Guan, Y. T., Zhu, W. P. (2008). Effect of biofilm on cast iron pipe corrosion in drinking water distribution system: Corrosion scales characterization and microbial community structure investigation. Corrosion Science 50, 2816 – 2823.

Teuber, M., Brodisch, K. E. U. (1977). Enzymatic activites of activated sludge. European Journal for Applied Microbiology 4, 185 – 194.

Thurman, R. B., Gerba, C. P. 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. Critical Reviews of Environmental Control 18, 295 – 315.

Thurnheer, T., Gmür, R., Guggenheim, B. (2004). Multiplex FISH analysis of a six-species bacterial biofilm. Journal of Microbiological Methods 56, 37 – 47.

Tielen, P. (2006). Einfluss extrazellulärer Enzyme auf die Struktur und die Eigenschaften von Biofilmen von *Pseudomonas aeruginosa*. Dissertation, University of Duisburg-Essen.

Tielker, D., Hacker, S., Loris, R., Strathmann, M., Wingender, J., Wilhelm, S., Rosenau, F., Jaeger, K.-E. (2005). *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiology 151, 1313 – 1323.

Titus, S., Gaonkar, S. N., Srivastava, R. B., Karande, A. A. (1995). Exopolymer production by a fouling marine bacterium *Pseudomonas alcaligenes*. Indian Journal of Marine Sciences 24, 45 – 48.

Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., Davison, M. (2001). Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1, 377 – 396.

TrinkwV, (Trinkwasserverordnung, German Drinking Water Ordinance; 2011). Umsetzung des § 18 TrinkwV 2001 in der behördlichen Praxis. Bundesgesetzblatt Teil I Nr. 21, 748 – 774.

Troch, P., Philip-Hollingworth, S., Orgambide, G., Dazzo, F. B., Vanderleyden, J. (1992). Analysis of extracellular polysaccharides isolated from *Azospirillum brasilense* wild type and mutant strains. Symbiosis 13, 229 – 241.

Tseng, T.-T., Tyler, B. M., Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiology 9, S2.

Umweltbundesamt (2011). Leitlinie zur hygienischen Beurteilung von Elastomeren im Kontakt mit Trinkwasser (Elastomerleitlinie). Empfehlung des Umweltbundesamtes, 1 – 52.

Underwood, G. J. C., Paterson, D. M., Parkes, R. J. (1995). The measurement of microbial carbohydrate exopolymers from intertidal sediments. Limnology and Oceanography 40, 1243 – 1253.

Ünlü, M., Morgan, M. E., Minden, J. S. (1997). Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis 18, 2071 – 2077.

Urbain, V., Block, J. C., Manem, J. (1993). Bioflocculation in activated sludge: an analytic approach. Water Research 27, 829 – 838.

Van der Aa, B. C., Dufrêne, Y. F. (2002). *In situ* characterization of bacterial extracellular polymeric substances by AFM. Colloids and Surfaces B: Biointerfaces 23, 173 – 182.

Van Hullebusch, E. D., Zandvoort, M. H., Lens, P. N. L. (2003). Metal immobilisation by biofilms: Mechanisms and analytical tools. Reviews in Environmental Science and Biotechnology 2, 9 – 33.

Van Ommen Kloeke, F., Geesey, G. G. (1999). Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs. Microbial Ecology 38, 201 – 214.

Veiga, M. C., Mahendra, K. J., Wu, W.-M., Hollingsworth, R. I., Zeikus, J. G. (1997). Composition and role of extracellular polymers in methanogenic granules. Applied and Environmental Microbiology 63, 403 – 407.

Vepsäläinen, M., Erkomaa, K., Kukkonen, S., Vestberg, M., Wallenius, K., Niemi, R. M. (2004). The impact of crop plant cultivation and peat amendment on soil microbial activity and structure. Plant and Soil 264, 273 – 286.

Vesy, C. J., Kitchens, R. L., Wolfbauer, G., Albers, J. J., and Munford, R. S. (2000). Lipopolysaccharide-binding protein and phospholipid transfer protein release lipopolysaccharides from Gram-negative bacterial membranes. Infection and Immunity 68, 2410 – 2417.

Vilain, S., Pretorius, J. M., Theron, J., Brözel, V. S. (2009). DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Applied and Environmental Microbiology 75, 2861 – 2868.

Vogt, M., Flemming, H.-C., Veeman, W. S. (2000). Diffusion in *Pseudomonas aeruginosa* biofilms: a pulsed field gradient NMR study. Journal of Biotechnology 77, 137 – 146.

Völker, S., Schreiber, C., Kistemann, T. (2010). Drinking water quality in household supply infrastructure – A survey of the current situation in Germany. International Journal of Hygiene and Environmental Health 213, 204 – 209.

Von Sengbusch, P., Mix, M., Wachholz, I., Manshard, E. (1982). FITC-labeled lectins and calcofluor white ST as probes for the investigation of the molecular architecture of cell surfaces. Studies on conjugatophycean species. Protoplasma 111, 38 – 52.

Vroom, J. M., De Grauw, K. J., Gerritsen, H. C., Bradshaw, D. J., Marsh, P. D., Watson, G. K., Birmingham, J. J., Allison, C. (1999). Depth penetration and detection of pH gradients in biofilms by two-photon excitation microscopy. Applied and Environmental Microbiology 65, 3502 – 3511.

Wagner, M., Assmus, B., Hartmann, A., Hutzler, P., Springer, N., Schleifer, K.-H. (1994). *In situ* analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Journal of Microscopy 176, 181 – 187.

Wagner, M., Amann, R. (1997). Molecular techniques for determining microbial community structures in activated sludge. In: Microbial community analysis. IAWQ Scientific and Technical Report No. 5, pp. 61 – 72. Cloete, T. E., Muyima, N. Y. O. (Eds.), IWA Publishing.

Waines, P. L., Moate, R., Moody, A. J., Allen, M., Bradley, G. (2011). The effect of material choice on biofilm formation in a model warm water distribution system. Biofouling 27, 1161 – 1174.

Waite, R. D., Rose, R. S., Rangarajan, M., Aduse-Opoku, J., Hashim, A., Curtis, M. A. (2012). *Pseudomonas aeruginosa* possesses two putative Type 1 signal peptidases, LepB and PA1303, each with distinct roles in physiology and virulence. Journal of bacteriology, published online ahead of print.

Walsh, S., Lappin-Scott, H. M., Stockdale, H., Herbert, B. N. (1995). An assessment of the matabolic activity of starved and vegetative bacteria using two redox dyes. Journal Microbiological Methods 24, 1 – 9.

Wang, H., Hu, C., Hu, X., Yang, M., Qu, J. (2012). Effects of disinfectant and biofilm on the corrosion of cast iron pipes in a reclaimed water distribution system. Water Research 46, 1070 – 1078.

Watnick, P., Kotler, R. (2000). Biofilm, city of microbes. Journal of Bacteriology 182. 2675 – 2679.

Wawrzynczyk, J., Szewczyk, E., Norrlöw, O., Dey, E. S. (2007). Application of enzymes, sodium tripolyphosphate and cation exchange resin for the release of extracellular polymeric substances from sewage sludge. Characterization of the extracted polysaccharides/glycoconjugates by a panel of lectins. Journal of Biotechnology 130, 274 – 281.

Webb, J. S., Nixon, M., Eastwood, I. M., Greenhalgh, M., Robson, G. D., Handley, P. S. (2000). Fungal colonization and biodeterioration of plasticized polyvinyl chloride. Applied and Environmental Microbiology 66, 3194 – 3200.

Webb, J. S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M., Kjelleberg, S. (2003). Cell Death in *Pseudomonas aeruginosa* Biofilm Development. Journal of Bacteriology 185, 4585 – 4592.

Weiner, R., Langille, S., Quintero, E. (1995). Structure, function and immunochemistry of bacterial exopolysaccharides. Journal of Industrial Microbiology 15, 339 – 346.

Wellinghausen, N., Köthe, J., Wirths, B., Sigge, A., Poppert, S. (2005). Superiority of molecular techniques for identification of Gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. Journal of Clinical Microbiology 43, 4070 – 4075.

Wentland, E. J., Stewart, P. S., Huang, C.-T., McFeters, G. A. (1996). Spatial Variations in growth rate within *Klebsiella pneumoniae* colonies and biofilms. Biotechnology Progress 12, 316 – 321.

Wetzel, R. G., Ward, A. K., Stock, M. (1997). Effects of natural dissolved organic matter on mucilaginous matrices of biofilm communities. Archives of Hydrobiology 139, 289 – 299.

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., Mattick J. S. (2002). Extracellular DNA required for bacterial biofilm formation. Science 295, 1487.

White, C., Gadd, G. M. (1996). Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration. Microbiology 1452, 2197 – 2205.

White, C., Gadd, G. M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. Microbiology 144, 1407 – 1415.

White, C., Gadd, G. M. (2000). Copper accumulation by sulphate-reducing bacterial biofilms. FEMS Microbiology Letters 183, 313 – 318.

Wilén, B. M., Jin, B., Lant, P. (2003). The influence of key chemical constituents in activated sludge on surface and flocculation properties. Water Research 37, 2127 – 2139.

Wilks, S. A., Michels, H., Keevil, C. W. (2005). The survival of *Escherichia coli* O157 on a range of metal surfaces. International Journal of Food Microbiology 105, 445 – 454.

Wimpenny, J. (2000). An overview of biofilms as functional communities. In SGM symposium 59: Community structure and co-operation in biofilms, pp. 1 – 24. Allison, D., Gilbert, P., Lappin-Scott, H., Wilson, M. (Eds.), Cambridge University Press, Cambridge.

Wingender, J., Neu, T., Flemming, H.-C. (1999). What are bacterial extracellular polymeric substances? In: Microbial extracellular polymeric substances, pp. 1 – 19. Wingender, J., Neu, T. and Flemming, H.-C. (Eds.), Springer Heidelberg, Berlin.

Wingender, J., Strathmann, M., Rode, A., Leis, A., Flemming, H.-C. (2001). Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*. Methods in Enzymology 336, 302 – 314.

Wingender, J., Jaeger, K.-E. (2002). Extracellular enzymes in biofilms. In: Encyclopedia of Environmental Microbiology, Vol. 3, pp. 1207 – 1223. Bitton, G. (Ed.), Wiley, New York.

Wingender, J., Flemming, H.-C. (2004). Contamination potential of drinking water distribution network biofilms. Water Science and Technology 49(11-12), 277 – 286.

Wingender, J. (2011). Hygienically relevant microorganisms in biofilms of man-made water systems. In: Biofilm Highlights. Springer Series on Biofilms, Vol. 5, pp. 189 – 238. Flemming, H.-C., Wingender, J., Szewzyk, U. (Eds.), Springer Heidelberg, Berlin.

Wingender, J., Flemming, H.-C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. International Journal of Hygiene and Environmental Health 214, 417 – 423.

Wloka, M., Rehage, H., Flemming, H.-C., Wingender, J. (2004). Rheological properties of viscoelastic biofilm EPS and comparison to the behavior of calcium alginate gels. Colloid and Polymer Science 282, 1067 – 1076.

Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D., Caldwell, D. E. (1994). Multicellular organization in a degradative biofilm community. Applied and Environmental Microbiology 60, 434 – 446.

Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D., Caldwell, D. E. (1998). *In situ* characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. Microbial Ecology 35, 213 – 223.

Wood, P. J. (1980). Specificity in the interaction of direct dyes with polysaccharides. Carbohydrate Research 85, 271 – 287.

Wozniak, D. J., Ohman, D. E. (1991). *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. Journal of Bacteriology 173, 1406 – 1413.

Wrangstadh, M., Conway, P. L., Kjelleberg, S. (1986). The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. Archives of Microbiology. 145, 220 – 227.

Wu, J., Xi, C. (2009). Evaluation of different methods for extracting extracellular DNA from the biofilm matrix. Applied and Environmental Microbiology 75, 5390 – 5395.

Wuertz, S., Pfleiderer, P., Kriebitzsch, K., Späth, R., Griebe, T., Coello-Oviedo, D., Wilderer, P. A., Flemming, H.-C. (1998). Extracellular redox activity in activated sludge. Water Science and Technology 37(4-5), 379 – 384.

Wuertz, S., Spaeth, R., Hinderberger, A., Griebe, T., Flemming, H.-C., Wilderer, P. A. (2001). A new method for extraction of extracellular polymeric substances from biofilms and activated sludge suitable for direct quantification of sorbed metals. Water Science and Technology, 43(6), 25 – 31.

Wullings, B. A., van der Kooij, D. (2006). Occurrence and genetic diversity of uncultured *Legionella* spp. in drinking water treated at temperatures below 15°C. Applied and Environmental Microbiology 72, 157 – 166.

Wüthrich, K. (2001). The way to NMR structures of proteins. Nature Structural Biology 8, 923 – 925.

Xu, K. D., Stewart, P. S., Xia, F., Huang, C. T., McFeters, G. A. (1998). Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Applied and Environmental Microbiology 64, 4035 – 4039.

Yarmolinsky, M. B. (1995). Programmed cell death in bacterial populations. Science 267, 836 – 837.

Yu, J., Kim, D., Lee, T. (2010). Microbial diversity in biofilms on water distribution pipes of different materials. Water Science and Technology 61(1), 163 – 171.

Zhang, Z.-J., Chen, S.-H., Wang, S.-M., Luo, H.-Y. (2011). Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress. Applied Microbiology and Biotechnology 89, 1563 – 1571.

Zieske, L. R. (2006). A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. Journal of Experimental Botany 57, 1501 – 1508.

Zita, A., Hermansson, M. (1997). Determination of bacterial cell surface hydrophobicity of single cells in culture and in wastewater *in situ*. FEMS Microbiology Ecology 152, 299 – 306.

APPENDIX

Publikationsliste

Referierte Publikationen

Michalowski, W. D., Flemming, H.-C., Wingender, J. (in prep.). Isolation and analysis of EPS from drinking-water biofilms - a critical assessment of common EPS isolation methods.

Vorträge

Alfer, B., Michalowski, W. D., Flemming, H.-C., Wingender, J. (2011). Dynamics of microbial populations and biochemical composition of drinking-water biofilms. Vortrag und Beitrag im Tagungsband der IWA Biofilms Conference 2011 - Processes in Biofilms, Shanghai/China.

Poster

Jachlewski, S., Michalowski, W. D., Mayer, B., Albers, S.-V., Wingender, J., Siebers, B. (2011). Hot Biofilms - EPS analysis of the archaeon *Sulfolobus acidocaldarius*. Poster und Beitrag im Tagungsband der IWA Biofilms Conference 2011 - Processes in Biofilms, Shanghai/China.

Alfer, B., Michalowski, W. D., Flemming, H.-C., Wingender, J. (2011). Dynamik der mikrobiellen Population und biochemischen Zusammensetzung von Trinkwasserbiofilmen. Poster und Beitrag im Tagungsband der Wasser 2011 - Jahrestagung der Wasserchemischen Gesellschaft, Norderney.

Michalowski, W. D., Flemming, H.-C., Wingender, J. (2010). Isolation of extracellular polymeric substances from drinking-water biofilms - a critical assessment of standard isolation methods. Poster und Beitrag im Tagungsband der Biofilms IV Konferenz, Winchester/UK.

Jachlewski, S., Michalowski, W. D., Wingender, J., Siebers, B. (2010). Hot Biofilms - EPS analysis of the archaeon *Sulfolobus acidocaldarius*. Poster und Beitrag im Tagungsband der Biofilms IV Konferenz, Winchester/UK.

Michalowski, W. D., Flemming, H.-C., Wingender, J. (2009). Isolation and analysis of extracellular polymeric substances from drinking-water biofilms. Poster und Beitrag im Tagungsband der 5th ASM Conference on Biofilms, Cancun/Mexico.

Michalowski, W. D., Bressler, D., Flemming, H.-C., Wingender, J. (2009). Proteine als Hauptbestandteile der EPS von Trinkwasserbiofilmen. Poster und Beitrag im Tagungsband der Wasser 2009 - Jahrestagung der Wasserchemischen Gesellschaft, Stralsund.

Michalowski, W. D., Bressler, D., Broekman, S., Flemming, H.-C., Wingender, J. (2008). Extracellular polymeric substances in drinking-water biofilms. Poster und Beitrag im Tagungsband der Biofilms III Konferenz in Garching/München.

Lebenslauf

"Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten"

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Composition, dynamics and function of extracellular polymeric substances in drinking-water biofilms"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im August 2012

(Witold Dariusz Michalowski)